Uromodulin: Biomarker for Kidney Tubular Function

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Declaration

I hereby declare that this dissertation is the result of my own work and effort. Wherever contributions of others have been involved or other sources of information have been used, they are acknowledged.

This work was carried out under the supervision of Prof. Dr. med. Olivier Devuyst at the Institute of Physiology, University of Zurich, Zurich, Switzerland, from November 2011 to December 2016.

Publications presented in this work:

1- Determination of uromodulin in human urine: influence of storage and processing.

Youhanna S., Weber J., Beaujean V., Glaudemans B., Sobek J., Devuyst O. *Nephrol Dial Transplant*. 2014. 29 (1): 136-145.

2- Common UMOD Variants Influence Urinary and Plasma Levels and Biochemical Properties of Uromodulin

Youhanna S., Schaeffer C., Hülsmeier A., Hennet T., Vollenweider P., Köttgen A., Bochud M., Swiss Kidney Project on Genes in Hypertension (SKIPOGH) team, Cohort Lausanne (CoLaus) team, Rampoldi L., Devuyst O.

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3- Associations of Urinary Uromodulin with Clinical Characteristics and Markers of Tubular Function in the General Population.

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5- Validation of Surrogates of Urine Osmolality in Population Studies.

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Abstract

Uromodulin is the most abundant protein in urine under normal physiological conditions. This glycoprotein is secreted exclusively by the epithelial cells of the thick ascending limb of the loop of Henle. Despite an extensive and long history of research on uromodulin, a clear understanding of its physiological role is still lacking.

The first aim of this thesis was to develop and characterize an assay, which enables accurate measurements of uromodulin levels in the urine. We then investigated the effect of a variant in the promoter region of the *UMOD* gene on the biochemistry of this glycoprotein and its plasma levels. We finally tried to identify the clinical and biological factors modulating the levels of uromodulin in urine in the general population.

We were able to characterize a robust and cost-effective assay to measure uromodulin levels in the urine and established a protocol for sampling, storage and experimental processing. Studying the relationship between uromodulin levels, clinical and genetic factors in samples from Swiss and Canadian cohorts, we showed that uromodulin is a reliable biomarker for kidney tubular mass and function in the general population. We also showed that variants in the *UMOD* promoter in addition to influence the absolute levels of uromodulin production and secretion it also modulates its glycosylation pattern.

These results contribute significantly to our understanding of the physiological role of uromodulin and highlight its usefulness as a biomarker for kidney function analysis.

Zusammenfassung

Unter normalen physiologischen Bedingungen ist Uromodulin das am häufigsten vorkommende Protein im Urin. Dieses Glykoprotein wird ausschliesslich von den Epithelzellen des dicken aufsteigenden Teils der Henle-Schleife sezerniert. Trotz der ausgiebigen und langen Forschungsgeschichte um Uromodulin fehlt uns noch immer ein klares Verständnis seiner physiologischen Funktion.

Das erste Ziel dieser Dissertation war die Entwicklung und Charakterisierung eines Tests, welcher präzise Messungen von Uromodulinkonzentrationen im Urin ermöglicht. Anschliessend untersuchten wir den Effekt einer Form der Promotor-Region des *UMOD* Gens auf die Proteinbiochemie sowie zirkulierenden Plasmalevels. Letztendlich versuchten wir klinische und biologische Faktoren zu identifizieren, die den Uromodulinspiegel in der gesamten Bevölkerung beeinflussen.

Wir waren in der Lage ein robustes und kostengünstiges Testverfahren zu entwickeln um Uromodulinkonzentrationen im Urin zu messen und etablierten ferner ein Protokoll zur Probennahme, Probenlagerung und der experimentellen Verarbeitung. Wir zeigten mittels Studien über die Beziehung von Uromodulinkonzentrationen mit klinischen und genetischen Faktoren in schweizer und kanadischen Kohorten, dass Uromodulin als verlässlicher Biomarker für die Nieren(tubuläre)-Masse und -Funktion in der allgemeinen Bevölkerung genutzt werden kann. Wir bewiesen des Weiteren, dass genetische Varianten des *UMOD* Promotors neben der Beeinflussung der absoluten Produktions- und Sekretionsmenge von Uromodulin auch seine Glykosylierungsmuster modulieren.

Die hier gezeigten Resultate tragen zum weiteren Verständnis der physiologischen Rolle von Uromodulin bei und untermauern dessen Nutzen als Biomarker für Nierenfunktionsanalysen.

I. Introduction

1. Kidney

1.1 General structure

Kidneys are bean-shaped organs located in the dorsal part of the abdominal cavity, one on each side of the lumbar spine. They are part of the urinary tract, which also includes the ureters, the urinary bladder, and the urethra. Kidneys are embedded in a fibrous capsule, which protects them from mechanical injury. There are three distinctive regions visible with the naked eye in a kidney: (from outside to inside) the cortex, the medulla, and the renal sinus. The cortex and medulla both form the renal parenchyma and encompass millions of nephrons, which are the functional units of the kidney. The renal sinus is a large cavity that is located below the renal parenchyma and opens to the exterior through the hilum across which pass the renal artery and vein, lymphatics, nerve supply and ureter that carries the final urine from the kidney to the bladder (Gyton A. et al. 2006).

The kidney displays a highly organized structure adapted to its homeostatic role combining the essential processes of filtration, secretion and reabsorption. The smallest functional unit of the kidney, the nephron, comprises several specialized cell types deriving from various embryological lineages (Figure 1). About 1 million nephrons lie in each kidney. Kidneys are not able to regenerate new nephrons, therefore, the number of nephrons decreases with renal injury, disease and age. Every nephron contains a mass of glomerular capillaries called the glomerulus that contains a network of capillaries lined with fenestrated epithelial cells. The entire glomerulus is encased in Bowman's capsule. Large amounts of fluid are filtered through the glomerulus, then flowing within a long tubule made of differentiated segments in which reabsorption and secretion events change the composition of urine. The final parts of these tubules are interconnected to form the collecting ducts, which open into the renal pelvis.

1.2 Physiology

As an organ involved in excretory, metabolic and endocrine activity kidneys play an essential role in maintaining vital functions. Kidneys are the primary means for eliminating waste products and toxins from the bloodstream and excreting them through the urine. Due to their

extensive capacity to filter and reabsorb solutes and water, they regulate body's fluid status, electrolyte balance and acid-base balance. They also produce or activate hormones that are involved in erythrogenesis, Ca^{2+} metabolism and the regulation of blood pressure and blood flow (Figure 2).

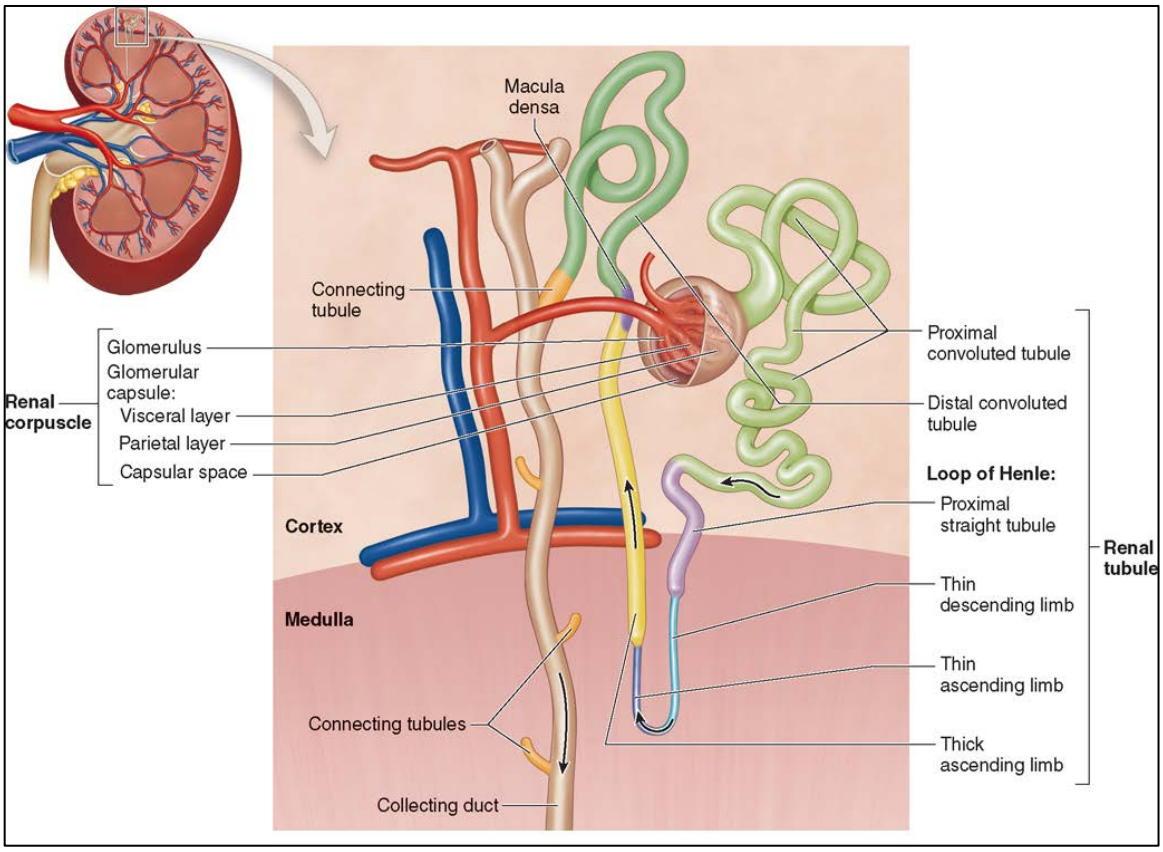

Figure 1: Structure of the nephron. Adapted from Junqueira's Basic Histology, 13e Chapter 19: The Urinary System.

Kidneys excrete around 1.5 L/day. They are perfused with large amount of ultrafiltrate (20 % of the cardiac output), which is considerably modified during tubular passage, and of urine thus leaving the body composition tightly affected by any changes in kidney function on one hand and exposing this organ to changes of the interior milieu unlike any other organ (Eckardt KU. et al. 2013).

The glomerular filtration barrier is made up of three sequential layers: the glomerular fenestrated endothelial cells, the glomerular basement membrane and the podocytes with their

foot processes enwrapping the glomerular capillaries (Haraldsson B. et al. 2008). Water, small solutes, and low-molecular-weight proteins (LMW) (up to the mass of albumin, 66.5 kDa) are capable of crossing the filtration barrier unlike plasma proteins with a mass of more than 60-70 kDa, especially if they are negatively charged. Thus, the ultrafiltrate leaving the glomerulus contains plasma solutes and several grams of LMW. The composition and the amount of this filtrate depend on numerous factors including the glomerular filtration rate (GFR), the product of the filtration area, the hydraulic permeability and the net ultrafiltration pressure. Alterations in glomerular haemodynamics or composition cause decreased GFR or increased leakage of proteins into the urine, which are both typical symptoms of renal disease.

As the glomerular filtrate gets into the renal tubules, it circulates progressively through the consecutive part of the tubule. Urine composition is affected by numerous mechanisms that are mediated by polarized transport systems in the epithelial cells lining the tubules. Throughout this course, a series of modifications takes place such as the massive reabsorption of LMW proteins, solutes, and water, secretion of a gel-like protein called uromodulin (Tamm-Horsfall protein) and elimination of excess potassium, acids and bases (Figure 1). In the proximal tubule, about two-thirds of the filtered solutes and water is reabsorbed. The cells lining the proximal tubule are equipped with multiligand receptors to carry out the endocytic uptake of filtered proteins (hormones, carrier proteins and enzymes) to be subsequently metabolized, as the human urine is protein-free under physiological conditions. This excessive protein uptake is essential for metabolic clearance, hormone homeostasis, and conversation of necessary vitamins (vitamin D, vitamin A, and vitamin B12); it also grants a milieu devoid of proteins for the cells lining distal nephron segments (Christensen EI. et al. 2009). In the last part of the proximal tubule, specific transporters drive the secretion of organic molecules and drug metabolites into the urine. Moreover, paracellular reabsorption of Ca^{2+} and Mg^{2+}occurs in the loop of Henle under the control of Ca^{2+}/ Mg^{2+} sensing receptors (Ferrè S. et al. 2012). Additionally, this segment of the tubule plays a crucial role in urine concentration by creating a hypertonic milieu in the medulla. The macula densa has a special structure of particular epithelial cells, located at the intersection between the thick ascending limb of the loop of Henle and the distal nephron. It regulates the glomerular blood flow through a mechanism called tubuloglomerular feedback by detecting the tubular NaCl concentration and interacting with the granular cells containing renin in the afferent arterioles (Singh P. et al. 2010). Lastly, the distal nephron, comprising the distal convoluted

tubule, the connecting tubule, and the collecting duct is susceptible to aldosterone and vasopressin (role discussed below) thus regulates the final urine composition and concentration. Many of the abovementioned tubular transport processes can be altered by specific drugs such as diuretics, aquaretics, calcimimetics, and the more recently developed inhibitors of glucose reabsorption (Reilly R. et al. 2000).

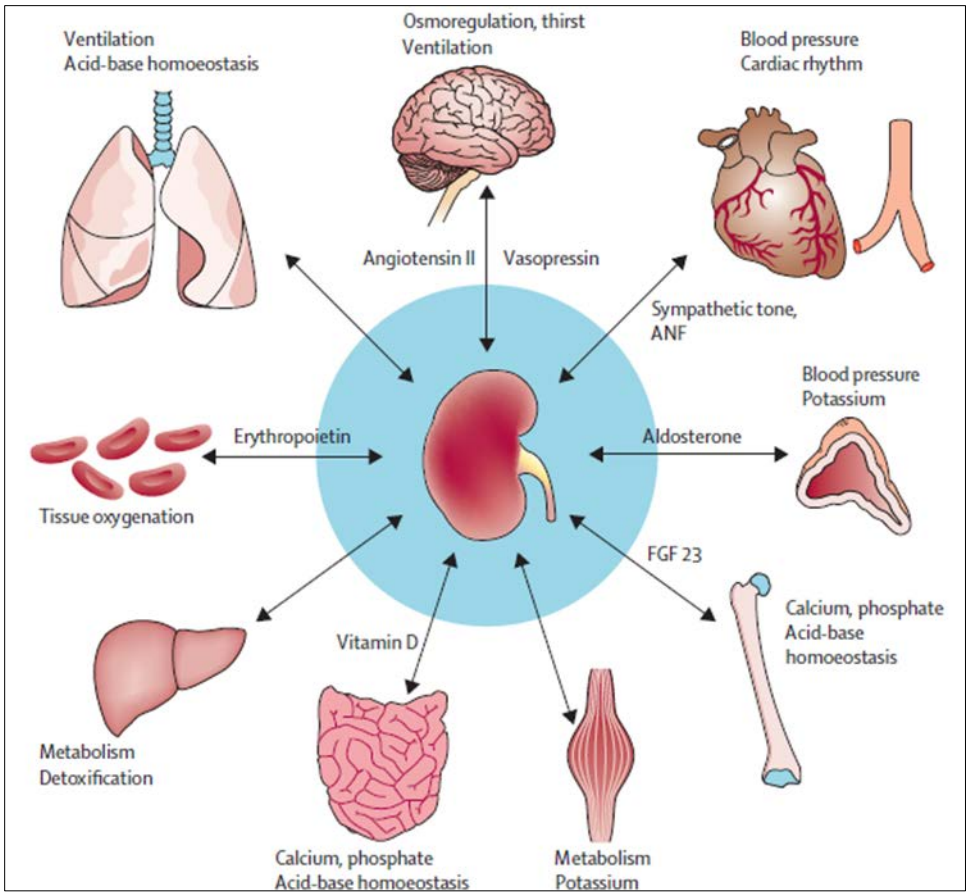

Figure 2: Effect of kidney function on essential homoeostatic processes. FGF: fibroblast growth factor. ANF: atrial natriuretic factor. Adapted from Eckardt KU. et al. 2013.

NaCl excretion, a critical solute for extracellular fluid volume and blood pressure regulation, is controlled by the renin-angiotensin-aldosterone system, the atrial natriuretic peptide, the sympathetic nervous system and to a slighter extent by antidiuretic hormone arginine vasopressin. However, the excretion of potassium depends mostly on the distal tubular flow rate and on the release of aldosterone and its action on the principal cells of the distal nephron. Vasopressin is involved in controlling osmoregulation by acting on aquaporin-mediated transport of water (Stockand JD. et al. 2010; Schrier W. et al. 2008). A more complex interaction between

bone, parathyroid glands, intestine and tubular segments delineate the balance between phosphorus and calcium involving fibroblast growth factor (FGF), klotho, parathyroid hormone, and vitamin D. Moreover, kidneys manage tissue oxygen supply via hypoxia-inducible erythropoietin production in peritubular fibroblasts of the renal cortex a role that makes the kidneys the main site of systemic oxygen sensing (Kurosu H. et al. 2008; Martin A. et al. 2012).

1.3 Pathophysiology

Kidney disease occurs when the structure and/ or the function of this organ is altered. Even the slightest disorder can increase the risk of kidney failure or lead to a series of complications in other organ systems, especially the cardiovascular system. The clinical manifestations and disease progressions are diverse. Depending on the duration, kidney disease can be classified as acute (within days) or chronic. Acute kidney injury and chronic kidney disease are associated: acute kidney injury can evolve to chronic kidney disease, and chronic kidney disease amplifies the risk of acute kidney injury (Chawla L. et al. 2012).

1.3.1 Chronic kidney disease

Chronic kidney disease (CKD) is a complex and developing disease associated with high mortality and high cost of care. In the last years, CKD prevalence has increased because of the high incidence of risk factors such as cardiovascular diseases, diabetes, aging as well as the occurrence of acute kidney injury in the general population. The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines bring the focus on the estimation of GFR from serum creatinine and monitoring markers of kidney damage (primarily albuminuria). Additionally, the use of standardized assays for creatinine and more accurate equations to estimate GFR were incorporated (Levey AS. et al. 2006; Levey AS. et al. 2009). Furthermore, cystatin C levels were added as a variable to estimate GFR and prognosis (Inker LA. et al. 2012). In 2013, albuminuria measurement was acknowledged by the KDIGO as an important marker for prognosis (Miller G. et al. 2009; Matsushita K. et al. 2012). The severity of CKD can be classified into 5 stages (Table 1). Stage 5 or end-stage renal disease (ESDR) involves in most of the case renal replacement.

Stage of Chronic Kidney Disease of all Types		
Stage	**Qualitative description**	**Renal Function (ml/min/1.73 m^2)**
1	Kidney damage – normal GFR	≥ 90
2	Kidney damage – mild ⬇ GFR	60-89
3	Moderate ⬇ GFR	30-59
4	Severe ⬇ GFR	15-29
5	End-stage renal disease	<15 (or dialysis)

Table 1: The five stages of chronic kidney disease. Adapted from http://guidelines.diabetes.ca/browse/chapter 29

1.3.2 Acute kidney injury

Acute kidney injury (AKI) is known as a sudden decrease in kidney function. The clinical manifestation of AKI is characterized by a rapid decrease in GFR, leading to alteration in electrolyte and acid-base balance. In addition to disturbances of extracellular fluid volume, hindrance of nitrogenous waste products and in most cases a decline in urine output (Bellomo R. et al. 2004). There has been controversy about ways to best evaluate kidney function this included markers that reveal best the decline in renal function and their levels to distinguish normal from abnormal renal function. The AKI KDIGO guideline relies on previous attempts to identify the disorder and focus on deviations in serum creatinine or drop in urine output as indicators of direct injury to the kidney and acute damage of function. In contrary to chronic kidney disease, the definition of acute kidney injury does not comprise markers of impairment, but rather a thorough analysis of urinary biomarkers preceding the decrease in GFR (e.g. kidney injury molecule 1, neutrophil gelatinase-associated lipocalin, and interleukin 18), thus grant a possible early detection and treatment (Chawla L. et al. 2012).

1.3.3 Genetic predisposition to kidney disease

The genetic architecture of kidney diseases includes Mendelian (monogenic) and complex diseases. Mendelian diseases are rare in the general population. Mutations in a single gene are sufficient to cause the manifestation of the disorder. In this type of diseases, only individuals

with a mutation in the causative gene are at risk for expressing the disorder. In contrast, complex disorders are common in the general population. In these diseases, genetics has a significant role but the display of the disease is due to interaction between genetic and environmental risk factors. Therefore, complex disorders do not show distinct inheritance patterns as in Mendelian disorders (Pritchard JK. et al. 2001). Thanks to classic and next-generation sequencing, more than hundred genes have been associated with monogenic kidney diseases. A classic example of these disorders is autosomal-dominant polycystic kidney disease (ADPKD), the most frequent monogenic renal disorder (Hildebrandt F. et al. 2010). Identification of a single-gene mutation underlying renal disease in families has become easier with the access to the whole exome and whole-genome sequencing. Moreover, with these techniques, investigation of the added effect of rare mutations and common disease susceptibility variants could be of help in explaining high phenotype heterogeneity, which are observed in several monogenic renal disorders. Genetic and environmental components shape the predisposition to complex multifactorial diseases such as chronic kidney disease. Genome wide association studies (GWAS) and admixture linkage disequilibrium methods assisted in identifying predisposing variants. Also; they favored the identification of genomic regions that have increased association with intermediate traits in the general population such as GFR, albuminuria and CKD. Furthermore, certain genomic regions are well known to be linked to specific renal diseases such as non-diabetic end-stage renal disease or focal-segmental glomerulosclerosis (Chambers J. et al. 2010; Köttgen A. et al. 2010). It is speculated that genetic risk variants can have a range of consequences (from small to large effects) on disease severity and this idea is becoming more convincing. Uromodulin is a good example of this: single mutations in the uromodulin (*UMOD*) gene are responsible for monogenic kidney disease (known as uromodulin-associated kidney disease) (Hart TC. et al. 2002), whereas specific common variants in the same gene increase predisposition to CKD, hypertension and many other complex renal phenotypes in the general population (Köttgen A. et al. 2009; Devuyst O. et al. 2017). Several rare inherited disorders shed light on common disorders of renal function. Monogenic diseases causing disturbances in sodium handling in the kidney helped understanding blood pressure regulation and mechanisms of action of diuretics (Ji W. et al. 2008; Devuyst O. et al. 2008) whereas disorders affecting proximal tubule like Fanconi syndrome highlighted the role of receptor-mediated endocytosis in acquired proximal tubule dysfunction and disease progression (Devuyst O. et al. 2010). Advanced knowledge about the

genetic components in kidney disease helps in making treatment decisions. For instance, identifying a genetic cause for a disorder in glomerular filtration barrier would aid in preventing improficient exposure to steroid treatment among children with nephrotic disorder.

2. Biomarkers for renal and tubular function

2.1 Biomarker: properties and role

Biomarkers are biological parameters that can be objectively measured and evaluated. They reflect physiological processes, pathological mechanisms or pharmacological responses to a therapy. A good biomarker is easily and inexpensively measured from an accessible source (blood, urine, cerebral spinal fluid etc.) with high sensitivity and specificity favoring an early detection of a particular disease, stratification for a certain risk as well as treatment monitoring. Major efforts have been made to explore and identify better biomarkers for disease prognosis hence better care for people at high risk for disease occurrence (McMahon G. et al. 2013).

Urinary biomarkers have been conventionally used to diagnose kidney disease, such as the presence of albuminuria which is a result of glomerular damage. Urine remains a better source of biomarkers in comparison to plasma. It can be noninvasively and continuously collected and it comprises plasma proteins that were filtered through the glomerulus as well as proteins secreted by the kidneys. Thus the urinary proteome directly reflects the condition of the urinary system in addition to some changes in the plasma proteome. Therefore, urine is an ideal source to study urological diseases at the same time it conveys information about the status of the entire body. Identifying potential biomarkers diseases in the urine, as the main component of investigative medicine, has gained a lot of attention in the last few years. Present studies focus on urogenital diseases (chronic and acute renal injuries, transplant rejection, bladder and prostate cancer). In addition, changes in the urinary proteome were also shown to be related to some systematic diseases, such as diabetes and coronary artery disease. These efforts used identified pathophysiology of the disease to detect a potential biomarker that is verified in clinical trials afterwards. A new approach had emerged in biomarker discovery with the advancement of protein mass spectrometry where the discovery of biomarkers is done by large-scale profiling on the protein content of the urine. This work revealed the display of more than 1000 gene products and numerous peptide fragments of large proteins. Thus, mass spectrometry offers the possibility to determine a urinary protein excretion profile which is clinically useful in early detection and

classification of several diseases as well as prognosis and monitoring of specific treatment (Shao C. et al. 2011).

2.2 Available urinary biomarkers for renal function and disease

Many conventional and novel biomarkers have been acknowledged for kidney disease prognosis and diagnosis. In this section, we will only discuss urinary biomarkers; these biomarkers reside mainly in the protein phase of the urine that consists of insoluble (48 % sediments and 3 % exosomes of total urinary protein) and soluble (49 % of the total urinary protein) proteins (Zhou H. et al. 2006).

2.2.1 Conventional biomarkers

This category includes filtration markers, such as creatinine and cystatin C, as well as markers of kidney damage, like urine sediment abnormalities and albuminuria (Table 2).

Creatinine: It has been adopted as a marker of GFR since many years. It is considered as a good marker of glomerular filtration since it is not metabolized in the kidney, and is freely filtered in the glomerulus. Nevertheless, the use of this marker in CKD and AKI is limited by the non-GFR determinants of creatinine concentration such as the secretion by the proximal tubule, degradation by the gut bacteria and reabsorption in patients with very low urine and tubular flow rates and in the case of certain medications. There are other factors such as gender, age, race, muscle mass and many others that affect creatinine secretion thus limiting its use for GFR estimation. To estimate GFR (eGFR) accurately and to overcome inter-person variability few creatinine-based equations have been developed. Despite these improvements, all the equations allowing to calculate eGFR have drawbacks, by either overestimating or underestimating GFR (Sandilands E. et al. 2013).

Cystatin C: It is a cysteine protease inhibitor that gets fully degraded locally in the renal tubules after glomerular filtration and proximal tubular reabsorption. Unlike creatinine, cystatin C is not actively secreted through the kidney and has no significant extrarenal elimination; also it is not influenced by changes in muscle mass which makes it an excellent marker, superior to creatinine, for renal function and accurately estimating GFR. Additionally, the presence of cystatin C in the urine suggests some form of proximal tubular injury indicating urinary cystatin C as a potential marker of acute tubular injury. Cystatin C excretion, as other low molecular

weight (MW) urinary biomarkers, is affected by increasing levels of albuminuria because of the competitive inhibition of cystatin c uptake in the presence of albuminuria. Different assays and methods exist to measure cystatin C but all are limited by the significant interassay variation, with some assays performing better for the diagnosis of AKI and CKD (Edelstein C. et al. 2011; Nejat M. et al. 2012; Inker L. et al. 2012).

Urinary protein*:* Proteins of the soluble phase originate mainly from glomerular filtration. High MW proteins are effectively retained by the glomerular filter due to the low sieving coefficient. Despite this process, proteins that are abundant in plasma such albumin can still cross the glomerular filter into the lumen of the nephron. Furthermore, small proteins with MW below 60 kDa as well as peptides can freely pass through the glomerular filter. Considerable amount of the filtered proteins and peptides are removed from the lumen of the nephron by specific apical uptake processes including receptor-mediated endocytosis. Accordingly, any modification in the level of certain soluble protein in the urine may indicate a variation in its amount in the plasma, alteration in the glomerular filtration or changes in the uptake system by the proximal tubule. Consequently, a modified excretion rate of a specific protein may designate a systemic disorder, glomerular impairment or a pathological state (Christensen EI. et al. 2001; Christensen EI. et al. 2002).

	Uses	Limitations
Creatinine	• Glomerular filtration marker • eGFR estimation • Biomarker of acute and chronic reduced kidney function	• Variability in generation rates across individuals • Significant tubular secretion leading to overestimation of GFR • Significant extra-renal elimination • Tubular reabsorption in low urine flow states • Increases late after AKI
Cystanin C	• GFR estimation (plasma) • Biomarker of proximal tubular dysfunction (urine) • Biomarker of acute and chronic reduced kidney function	• Increases late after AKI • May increase in inflammatory states or in thyroid dysfunction independent of kidney function • Urinary Cystatin C is altered in the presence of albuminuria
Albuminuria	• Biomarker of glomerular filtration barrier dysfunction • Biomarker of proximal tubular dysfunction • Early biomarker of AKI • Independent risk factor for - cardiovascular - all-cause mortality- and ESRD	• 24 h collections unreliable • Significant intra-individual variability in albumin/ creatinine ratio over short time periods

Table 2: Use and limitations of conventional biomarkers. AKI: acute kidney injury; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease. Adapted from McMahon G. et al. 2013.

2.2.2 Novel biomarkers

Levels of conventional biomarkers increase relatively late during injury processes, thus preventing prognosis and limiting early intervention that might circumvent disease progression. Moreover, conventional biomarkers in most of the cases do not allow anatomical location of the injury; the majority of these biomarkers reflect glomerular function and proximal tubule activity whereas other segments of the nephron are underrepresented. Therefore, novel biomarkers are needed for early and specific identification of alterations in the different parts of the nephron (Figure 3). These novel biomarkers will allow precocious intervention, improved stratification

and good monitoring of treatment efficiency (McMahon G. et al. 2013). These biomarkers help identifying and classifying renal diseases with various clinical conditions depending on their anatomical expression. i.e. lipocaline-2 or neutrophil gelatinase-associated lipocalin (NGAL), Interleukin 18 (IL-18) and Kidney Injury Molecule 1 (KIM-1) are biomarkers for AKI; Asymmetric dimethylarginine (ADMA), β-trace protein (BTP), L-type fatty acid binding protein (L-FABP) are biomarkers for CKD; monocyte chemotactic peptide-1 (MCP-1) (combined with NGAL), α-1-acid glycoprotein, and ceruloplasmin for glomerular disease (Edelstein C. et al. 2011; Nejat M. et al. 2012; Ozer J. et al. 2010).

Lipocalin-2 (LCN2): it is a 21 kDa protein expressed primarily by immune cells, hepatocytes and renal tubular cells. It can be readily detected in the urine and is protease-degradation resistant. This makes it an ideal biomarker for AKI. LCN2 appears in the urine shortly after a hypoxic injury but once AKI is resolved, both plasma and urinary NGAL levels return to baseline. In addition to AKI, LCN2 could also be a convenient biomarker for CKD especially for identifying CKD patients at risk of a significant decline in GFR, since it is highly sensitive to subtle changes in tubular function. Urinary and serum LCN2 levels are increased in many kidney diseases including IgA nephropathy, autosomal dominant polycystic kidney disease and diabetic nephropathy (Mishra J. et al. 2003; Haase M. et al. 2011).

KIM-1: A transmembrane protein expressed at very low levels in normal kidney. Under ischemic condition or nephrotoxic AKI dedifferentiated proximal tubular cells increase the expression of KIM-1 and let the extracellular domain of KIM-1 appear in the urine succeeding ischemic injury. KIM-1 levels can be detected easily by urinary dipstick, making it a suitable and available biomarker of AKI. Moreover, increased urinary KIM-1 levels predict an elevated risk of mortality or necessity for dialysis in hospitalized patients with AKI. As a biomarker of AKI KIM-1 may be more reliable in patients with normal baseline kidney function, as is the case for many novel biomarkers. This could be due to its upregulation in many chronic kidney diseases with the degree of expression associated with tubulointerstitial fibrosis. Its role in CKD is under investigation (Han WK. et al. 2002; Bonventre J. et al. 2009).

IL-18: it is a pro-inflammatory cytokine expressed mainly by macrophages but also by monocytes, dendritic cells and kidney epithelial cells. It is implicated in the innate and adaptive immune reaction and is upregulated in inflammatory conditions. Due to its expression by kidney

tubular epithelium urinary IL-18 was suggested as a potential marker of AKI. It is believed that IL-18 is one of the mediators of injury in ischemic AKI as its interstitial expression was proved to increase in mouse models of AKI whereas IL-18 Inhibition has been accompanied by recovery from inflammatory disease. Moreover, in models of ischemia, it has been shown that mice deficient in caspase, an IL-18-activating enzyme, were less prone to develop acute tubular necrosis than wild-type mice. The early increase in IL-18 levels in patients with sepsis in the intensive care unit makes it a good predictor of AKI, particularly when combined with NGAL. IL-18 may be a more general marker of inflammation rather than a specific marker of AKI, mainly in elderly where baseline reduced kidney function underlies (Parikh CR. et al. 2004; Parikh CR. et al. 2006).

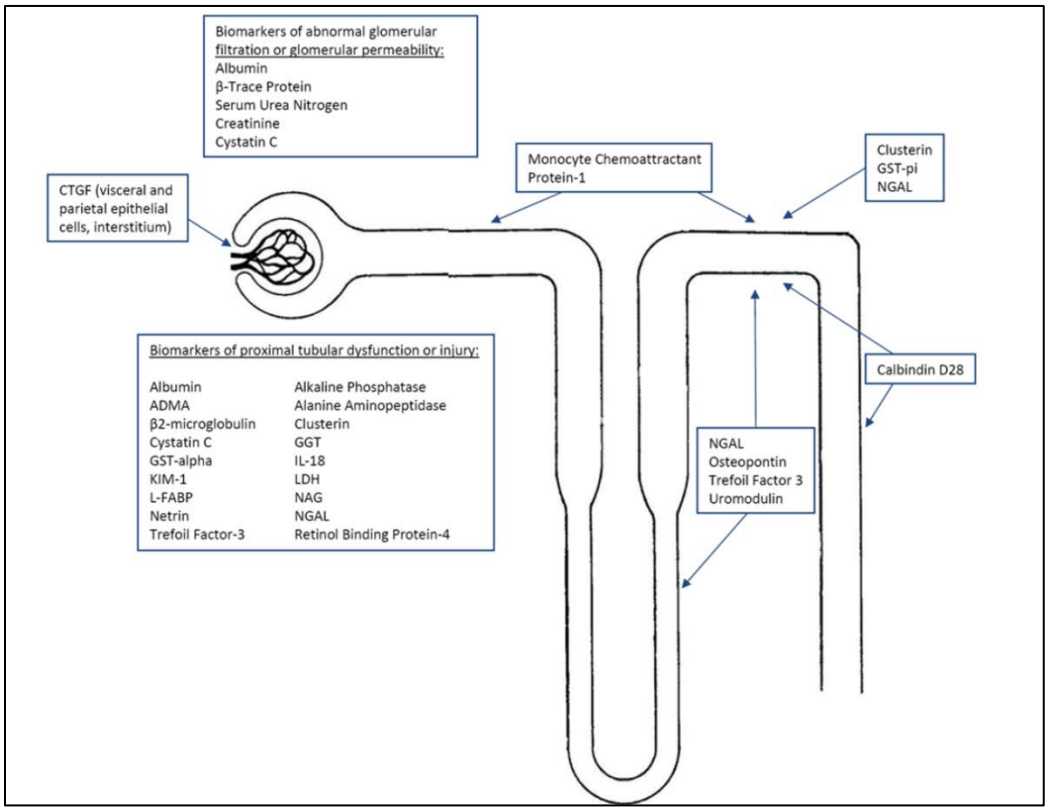

Figure 3: Anatomical localization of biomarkers along the nephron. Abbreviations: GST: Glutathione S-transferase; CTGF: Connective tissue growth factor; NGAL: Neutrophil gelatinase-associated lipocalin; ADMA: Asymmetric dimethyl arginine; KIM-1: Kidney injury molecule 1; L-FABP: Liver-type fatty acid binding protein; GGT: Gamma-glutamyl transpeptidase; IL-18: Interleukin 18; LDH: lactate dehydrogenase; NAG: N-acetyl glucosaminidase. Adapted from McMahon G. et al. 2013.

The joint efforts of basic science investigations and modern clinical epidemiology techniques helped to discover and validate novel biomarkers of kidney disease. Before largely implementing

the newly identified biomarkers in the clinic it is crucial to test those in larger studies with clinically important endpoints. It will be also required to compare conventional versus novel biomarkers for their added clinical utility as well as their correlation to the severity of disease with biomarker levels and duration of elevation. Additionally developing reliable assays will facilitate their measurements.

3. Uromodulin

Uromodulin has been first described in 1873 by Carlo Rovida and named as cilindrina. In 1950 Tamm and Horsfall described the same mucoprotein as they were able to purify it from urine and they reported its inhibitory effect to viral hemagglutination. Tamm-Horsfall protein is the most abundant protein in urine under physiological conditions and can form a gel-like structure (Devuyst O. et al. 2017).

3.1 Biology of the protein: Structure and biosynthesis

3.1.1 Structure and Maturation: The precursor of uromodulin has 640 amino acids before entering the endoplasmic reticulum (ER) where it gets glycosyl-phosphatidylinositol (GPI) anchored and glycosylated. After this step, it travels to the apical plasma membrane of epithelial cells where it gets cleaved to join the lumen. The processing step in the ER is the longest during protein maturation due to the complex tertiary structure attributed to the high number of cysteine residues (7 % of amino acid content) which are all involved in the formation of intramolecular disulfide bonds (Serafini-Cessi F. et al. 1993). The GPI-anchored protein has a molecular weight of 100 kDa (reduced condition) of which 30 % is credited to N-glycans. Confirmation of O-glycosylation has also been described (Easton RL. et al. 2000). The high glycan content provides the protein with several chemical and physical properties, thus various roles. Uromodulin is composed of several domains: a leader peptide that allows its entry into the secretory pathway, 4 epidermal growth factor-like domains (EGF-II and EGF-III are predicted to be calcium-binding domains) these modules are important for protein-protein interaction (Bokhove M. et al. 2016), a central domain with unknown function and it contains 8 conserved cysteines and a zona pellucida (ZP) domain that is essential for protein polymerization based on the ionic conditions (Jovine L. et al. 2002) (Figure 4).

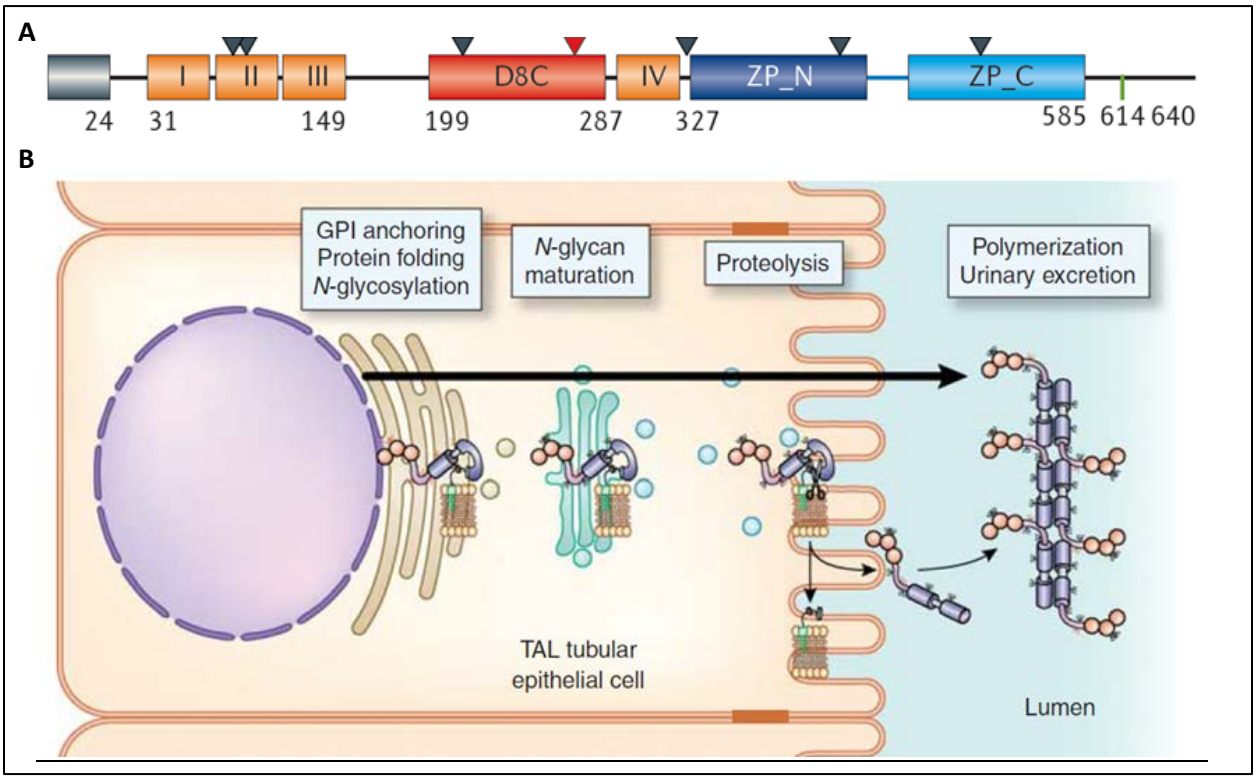

Figure 4: A: Uromodulin has a main peptide (grey), 4 epidermal growth factor (EGF)-like domains (orange), a cysteine-rich D8C domain of yet unknown function (red), bipartite zona pellucida (ZP_N and ZP_C) domain (blue), and glycosyl-phosphatidylinositol (GPI)–anchoring site at position 614 (green). The 7 N-glycosylation sites are indicated in triangles. The high-mannose chain site on residue Asn 274 is indicated with a red triangle. Adapted from Devuyst O. et al. 2017. B: Model of uromodulin maturation, excretion and polymerization. Uromodulin is produced by the epithelial cells of the TAL. In the endoplasmic reticulum it gets GPI-anchored, N-glycosylated and the disulfide bonds are formed. In the Golgi apparatus all N-glycosylated chains are modified except the one on Asn 274 that keeps the high-mannose moiety. On the plasma membrane polymerization-incompetent uromodulin is cleaved by a protease hepsin to join the lumen where it gets polymerized to form filaments. Adapted from Rampoldi L. et al. 2011.

3.2.1 Localization and Secretion: Uromodulin is exclusively expressed by the epithelial cells lining the thick ascending limb (TAL) of Henle's Loop. It is mainly detected at the apical plasma membrane, although localization at the basolateral side of TAL cells has also been reported (Bachmann S. et al. 1985; Peach RJ. et al. 1988). Studies on uromodulin trafficking in transfected polarized epithelial cells in addition to evidence its presence at very low concentrations in the blood compared to urine (ng vs µg) suggest its release from the basolateral side (Jennings P. et al. 2007). Uromodulin transcripts are documented from embryonic day 16.5 in the developing mouse kidney (Chen Y. et al. 2006) whereas in humans, the protein is

identified from gestational week 16 by immunohistochemistry analysis and from week 20 in the amniotic fluid (Zimmerhackl LB. et al. 1996). Its secretion increases progressively with time and maturation of TAL tubules until birth. In mature TAL cells, uromodulin is the most abundant transcript and has a short half-life (about 9 h in rabbit and 16 h in humans) (Grant M. et al. 1973; Serafini-Cessi F. et al. 2003). A person can produce approximately between 50-150 mg/day under physiological conditions (Chabardes GD. et al. 2003). A conserved proteolytic cleavage is essential to release uromodulin from the apical plasma membrane into the tubule's lumen (Santambrogio S. et al. 2008) and is crucial for protein polymerization since it releases an inhibitory motif that prevents premature protein assembly (Schaeffer C. et al. 2008). The protein is released in the lumen after cleavage by type II transmembrane serine protease hepsin (Brunati M. et al. 2015) where it can form a network of filaments and small fibrils with a width of about 100 Å and average length of 25,000 Å that tend to aggregate (Porter K. et al. 1955). Filaments of uromodulin are arranged in a double helix with a diameter of 90 to 120 Å (Jovine L. et al. 2002).

3.1.3 Expression and Conservation: Uromodulin is present in the kidney of all mammals. *UMOD* is located on chromosome 16p12.3-16p13.11 and is spread over 20 Kb it has 11 exons (exon 1 is non-coding). Its transcription is driven by a promoter (3.7 kbp), of which a large part (the first 589 bp) is highly conserved in mammalian species (Pook MA. et al. 1993). It is suggested that this region harbours the required *cis*-regulatory elements for UMOD expression in the nephron. Studies have proved that the 5' proximal flanking region of *UMOD* is highly conserved between mice, rats and humans (Zhu X. et al. 2002). *In silico* exploration of the upstream region of *UMOD* showed numerous conserved binding motifs among studied primates and rodent species suggesting relevant transcription factors expressed in the kidney that might regulate the expression of uromodulin (Srivastava R. et al. 2014). *UMOD* gene is CpG rich but not in the promoter region which implies a possible epigenetic regulation of uromodulin (Rosenbloom K. et al. 2015). There is a similarity between uromodulin sequence and domain composition with one of glycoprotein-2 (GP-2) that is the major component of zymogen granule membranes of exocrine pancreas, and liver-specific ZP domain-containing protein. Genes of both, GP-2 and *UMOD* lie adjacent on chromosome 16p12.3, suggesting that they could have evolved from a common ancestral gene (Fukuoka S. et al. 1992) (Figure 5). Like uromodulin (see below), GP-2 is capable of binding *Escherichia Coli* (*E. Coli*) of the fimbriated type I, proposing that both proteins have similar protective functions against microorganisms in the

urinary and digestive system (Yu S. et al. 2009). Anti-uromodulin immunoreactivity was reported in the kidneys of mammals, in layers of the skin of several amphibians and fishes, and in the distal tubules of the kidney of some amphibians (Howie AJ. et al. 1993). Comparative genomic analysis acknowledges UMOD homologs in amphibian (*Xenopus tropicalis*) and fish (*Danio rerio*) genomes, with significant sequence similarity at the predicted protein level. The function of these homologs and their relevance for comparative physiology remain to be determined.

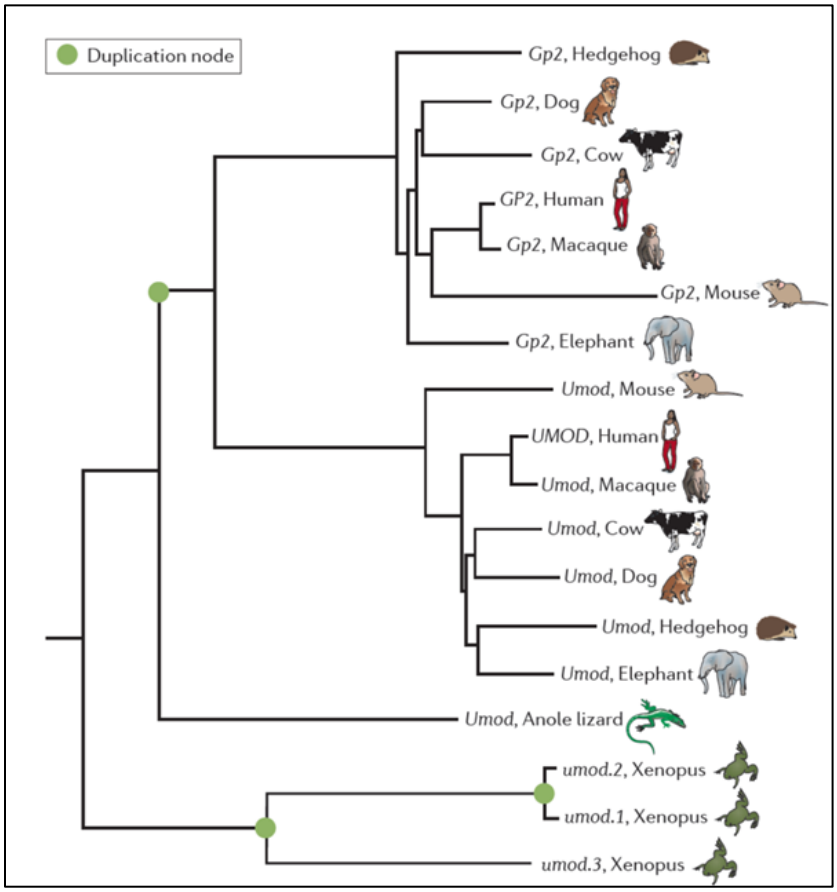

Figure 5: Evolution of *UMOD* gene. Phylogenic tree shows the assumed evolutionary relationship between uromodulin and glycoprotein 2. Adapted from Devuyst O. et al. 2017.

3.2 Physiology of uromodulin

The exact physiological role of uromodulin is still to be defined. However, multiple investigations have suggested that the glycoprotein may have many physiological functions

(Figure 6) due to its highly variable post-translational modifications, GPI-anchoring and multiple domains.

3.2.1 Water homeostasis*:* The particular formation of filamentous gel-like structures of uromodulin on the luminal plasma membrane of epithelial cells in the TAL may serve as a physical barrier (polyanionic gel) to water permeability and can be implicated in maintaining the countercurrent gradient in the interstitium. Additionally, *Umod*-knockout mice exhibited urine concentration disabilities after water deprivation (Bachmann S. et al. 2005).

3.2.2 Salt handling and blood pressure regulation: Uromodulin may have a role in water/ electrolyte balance in the TAL, this hypothesis is supported by Mutig et al. In his work Mutig et al. showed that intracellular trafficking of furosemide-sensitive Na-K-2Cl cotransporter (NKCC2) to the apical surface of the TAL is enhanced by uromodulin: An increase in uromodulin production is associated with increased NKCC2 expression and sodium reabsorption (Mutig K. et al. 2011) a fact evidenced by Trudu et al. (Trudu M. et al. 2013). Moreover, Renigunta et al. confirmed the significant increase of the activity of renal outer medullary potassium channel (ROMK2) with the expression of uromodulin. There is a direct interaction and positive regulation of its delivery to the plasma membrane. In another data set, it was highlighted that the lack of uromodulin in *Umod*-knockout mice resulted in a significant upregulation of ROMK2, a consequence of a reduction of the number of channels at the plasma membrane and their increase in the vesicular pool (Renigunta A. et al. 2011). Further investigation is needed to prove the specificity of this effect on ROMK since ion transporters of downstream segments (Na-Cl cotransporter, a-epithelial Na channel) were also found to be significantly upregulated in uromodulin-deficient mice (Bachmann S. et al. 2005).

3.2.3 Protection against urinary tract infection (UTI): Uromodulin gel-like structure may also be involved in other advantageous functions such hindering the epithelial binding of uropathogenic bacterial in UTI. N-linked high mannose chains of uromodulin compete for the binding with uroplakin receptors from *E.Coli* and prevent their adherence to glycoproteins and glycolipids of the luminal plasma membrane thus conferring protection against UTI (Chabardes GD. et al. 2003). Mo. et al. showed that *Umod*-knockout mice have increased susceptibility to UTIs (Mo L. et al. 2004). In humans, Wolf et al. reported recurrent UTIs in children with *UMOD* mutations (Wolf MT. et al. 2006), whereas as Garimella et al. showed an association between

high urinary uromodulin levels and lower risk for UTI in older community-dwelling adults independent of traditional UTI risk factors (Garimella S. et al. 2016).

3.2.4 Protection against stone formation: It has also been suggested that uromodulin reduces the aggregation of calcium oxalate crystals via its sialylated and negatively charged glycans thus acts as an inhibitor of urinary stone formation. In *Umod*-knock-out mouse models an increased tendency for calcium crystal formation and a reduced ability to inhibit the adhesion of calcium oxalate monohydrate crystals to renal epithelial cells has been documented. Similarly, uromodulin deficiency in humans may be a risk factor for nephrolithiasis (Grant A. et al. 1973; Serafini-Cessi F. et al. 2003). In addition to its inhibitory role for crystal formation, uromodulin might influence the paracellular handling of calcium in the TAL (since it can modulate TAL activity). It can also stabilize the calcium channel transient receptor potential cation channel subfamily V member 5 (TRPV5) in the DCT thus potentially influencing the urinary concentration of calcium phosphate, calcium oxalate as well as calcium excretion (Wolf MT. et al. 2013).

3.2.5 Immunomodulation and protection against acute kidney injury: Another protective role has been attributed to uromodulin in acute kidney injury by decreasing inflammation and improving recovery. *Umod*-knockout mice evidenced more functional and histological kidney damage and showed late recovery after ischemia-reperfusion injury in comparison to wild-type mice. In wild-type animals exposed to ischemia-reperfusion injury uromodulin accumulated at the basolateral side of TAL cells that is in close contact with cells from the proximal S3 segment. This observation highlights a protective role of UMOD mediated by cross-talk between segments possibly by binding and inhibiting components of the immune system via N-linked-carbohydrate sequences (El-Achkar TM. et al. 2011). The same group showed a downregulation in uromodulin expression at the peak of acute kidney injury, but an upregulation 48 h after ischemia-reperfusion injury. These findings need to be validated in humans (El-Achkar TM. et al. 2008). Of note, the GPI-enriched domain of UMOD may also have a receptor-like function in endocytosis which may implicate a role in signal transduction and cell surface event modulation (Sabharanjak S. et al. 2002). Finally, several *in vitro* studies proved that uromodulin can bind, interact with and activate immunity-related molecules, such as immunoglobulin G, complement 1q, and tumor necrosis factor α (Rhodes DC. et al. 1993; Rhodes DC. 2000; Hession C. et al. 1987), indicating that uromodulin may play a role in innate immunity of the kidney. Also, through interacting with

and activating components of the immune system, monocytes, neutrophils, and myeloid dendritic cells via toll-like receptor 4 uromodulin can act as a chemoattractant as well as a proinflammatory molecule (Säemann MD. et al. 2005; Schmid M. et al. 2010). Tubulointerstitial nephritis was induced in rabbits, rats, and mice after uromodulin supplementation (Mayrer R. et al. 1982; Hoyer JR. 1980). Anti-uromodulin antibodies were observed in mice, as a result of toll-like receptor 4 functions (Säemann MD. et al. 2005). Taking together the above- mentioned facts evidence an association between interstitial uromodulin release and inflammatory cell infiltration as well as the increase in uromodulin-specific autoantibodies in several inflammatory disorders and infections of the urinary tract (Säemann MD. et al. 2005). This indicates that uromodulin could act as a danger-sensing molecule which can trigger an inflammatory response in the presence of potential injury or damage to the nephron integrity. Nevertheless, the proinflammatory role of uromodulin remains debatable. El-Achkar et al. proved that *Umod*-knockout mice develop more functional and histological renal damage after ischemia-reperfusion injury compared to wild-type animals (El-Achkar TM. et al. 2008).

3.2.6 Cast nephropathy: In a disorder context, uromodulin constitutes a milieu for hyaline cast and proteinaceous cast, which are associated with low-molecular-weight proteinuria (Sanders PW. et al. 1990). The interaction between uromodulin and free light chains results in the precipitation of the latter in the distal tubule evolving into casts that cause obstruction and tubulointerstitial damage (Ying WZ. et al. 2012).

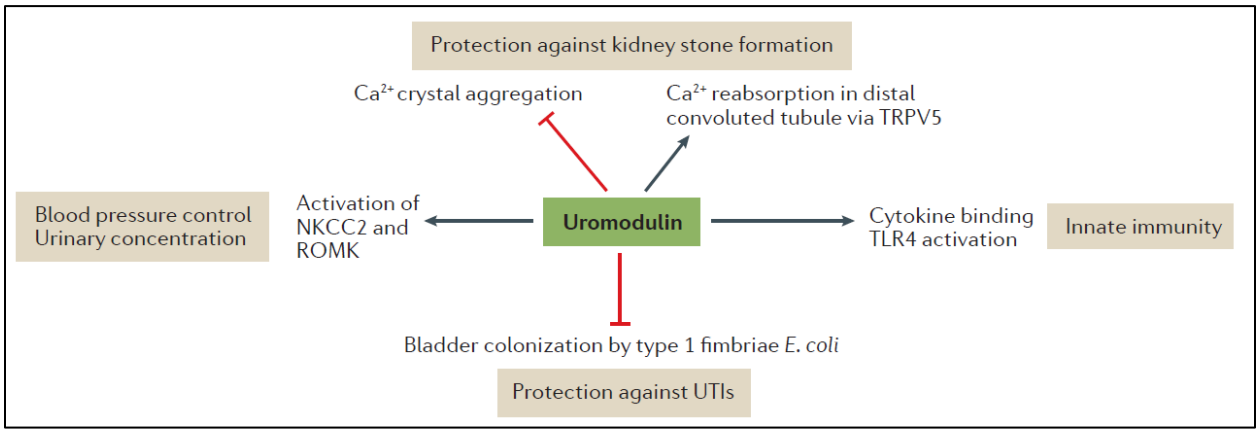

Figure 6: proposed physiological roles of uromodulin. Animal studies using *Umod*-knockout mice suggested that uromodulin modulates blood pressure, urine concentration and might play a role in protection against urinary tract infections and stone formation (UTIs) as well as in activating the immune system.

NKCC2: sodium–potassium–chloride co-transporter, ROMK: Renal outer medullar potassium channel, TLR4: Toll-like receptor 4. TRPV5: transient receptor potential cation channel. Adapted from Devuyst O. et al. 2017.

3.2.7 Uromodulin as a biomarker

3.2.7.1 Congenital disorder: Urinary uromodulin has been suggested as an *in utero* biomarker for fetal tubular development and to predict AKI of newborns since it is secreted by human fetal tubul but cannot cross the placental barrier. In prenatal Bartter cases, uromodulin is dramatically reduced when compared to levels from cases with postnatal Bartter (Zimmerhackl LB. et al. 1996; Askenazi D. et al. 2016; Schröter J. et al. 1993).

3.2.7.2 Tubular function and eGFR: Previous separate studies have shown decreased levels of uromodulin in subjects with tubular damage, in the presence of ADPKD as well as in tubulointerstitial nephritis (Lynn KL. et al. 1984; Thornley C. et al. 1985; Tsai C. et al. 2000). Moreover, in recent investigations Ledo et al. presented in an expression study a strong correlation between uromodulin mRNA expression levels and eGFR values in CKD vs control subjects while Garimella et al. described in a study involving elderly that a decrease in uromodulin levels indicates a risk for progressive renal disease and mortality (Ledo N. et al. 2015; Garimella PS. et al. 2015).

3.2.7.3 Acute kidney injury (AKI): In a clinical study, investigating AKI onset after cardiac surgery uromodulin has been suggested as a biomarker for AKI. Low uromodulin levels were associated with elevated risk for AKI in post cardiac surgery subjects implying that uromodulin levels might reflect poor tubular health at baseline, or suggesting a protective role of uromodulin against AKI (El‑Achkar TM. et al. 2008; Garimella PS. et al. 2017).

3.3 Pathophysiology of uromodulin

3.3.1 Monogenic disorders: Mutations in the *UMOD* gene have been shown to cause medullary cystic kidney disease type 2, familial juvenile hyperuricemic nephropathy and glomerulocystic kidney disease which are collectively referred to autosomal dominant tubulointerstitial kidney disease uromodulin-related (ADTKD-UMOD) with an estimated prevalence of 1:100.000 (www.orpha.net). Incomplete penetrance and *de novo* mutations may explain the absence of a family history in some patients (Hart TC. et al. 2002). So far, 125 *UMOD* mutations have been described of which 120 are missense mutations. 60 % of the total mutations affect the cysteine residues. Most of these mutations found in exons 3 and 4 and occur in the N-terminal half of the

protein (Devuyst O. et al. 2017). These mutations have a significant impact on the maturation and the trafficking of uromodulin causing its retention in the ER along with decreased expression at the plasma membrane, and declined secretion into the tubular lumen (Rampoldi L. et al. 2003). The intracellular buildup of the misfolded mutant protein in the ER of TAL cells results in cellular damage that can stimulate an immune response translated by immune cell infiltration and production of pro-fibrinogenic lymphokine in the tubule interstitium (Vyletal P. et al. 2006; Bernascone I. et al. 2010). Schaeffer et al. showed in a recent work that mutant UMOD retained in the ER and degraded via the proteasome pathway interacts with several ER chaperones, implying that uromodulin crosses the calnexin cycle to be properly folded. By transcriptional profiling, they proved that expression of mutant uromodulin mainly affects ER function, together with protein folding and ER-associated degradation (ERAD) and induces stress response. They also showed that ER stress activates the UPR perturbing ER homeostasis and thus reducing the amount of protein entering the ER while increasing ER folding capacity and ERAD (Schaeffer C. et al. 2017). These studies demonstrate that ADTKD-UMOD is an ER storage disease. Despite these findings, a clear mechanism of the progressive tubulointerstitial injury in ADTKD-UMOD is not well-understood yet. All ADTK diseases exhibit interstitial fibrosis, tubular atrophy and dilation, thickening and lamellation of tubular basal membranes. A typical characterization of ADTKD-UMOD is the decrease in urate fractional excretion causing hyperuricaemia and often gout. ADTKD-UMOD has a heterogeneous clinical presentation of symptoms, age at onset, presence of cysts, and rate of progression to end-stage renal disease. Currently, the only available treatment is renal replacement therapy (Bollée G. et al. 2011; Eckardt KU. et al. 2015).

Uromodulin has been associated with various renal defects: It has been found to accumulate in cast nephropathy, to deposit in the renal interstitium in reflux nephropathy, renal allografts rejection and interstitial diseases. UMOD deposits were in some cases associated with inflammatory infiltrate (Resnick JS. et al. 1978; Zager RA. et al. 1978; Cohen AH. et al. 1984).

Furthermore, a positive correlation has been reported between uromodulin urinary excretion and eGFR. Reduced levels of uromodulin were observed in numerous conditions affecting kidney function and/or integrity, such as glomerulonephritis, diabetes nephropathy, lupus nephritis, tubulointerstitial nephropathy, and polycystic kidney disease. Of note, a shorter atypically

processed urinary uromodulin fragments were detected in Fabry disease patients (Vyletal P. et al. 2008; Vyletal P. et al. 2010).

3.3.2 Complex disorders: In addition to its direct involvement in ADTKD-UMOD, recent GWAS pointed at uromodulin as a risk factor for CKD, kidney stones, and hypertension (Rampoldi L. et al. 2011). Lately, Köttgen et al. have carried out an influential work on UMOD that contributed to redraw the attention of scientists to this protein. In her GWAS, performed in a population-based cohort, mainly of European ancestry, Köttgen et al. reported a single nucleotide polymorphism (SNP) mapping within the promoter region of *UMOD* gene that is associated with eGFR and increased risk for CKD (Köttgen A. et al. 2010). The minor allele T of the identified SNP rs12917707 (G/T) confers 20 % reduction in CKD risk and elevated eGFR. The reported results were independent of known risk factor for renal disease (presence of hypertension or diabetes). This outcome was validated in large European cohorts, Icelandic population, Africans, East Asians as well as in European isolates (Chambers JC. et al. 2010; Pattaro C. et al. 2012; Sveinbjornsson G. et al. 2014; Liu CT. et al. 2011; Okada Y. et al. 2012). Replication studies revealed additional association between a second *UMOD* variant rs4293393 (located in the promoter region of *UMOD* gene and in full linkage disequilibrium (LD) with rs12917707) and risk for CKD and kidney function (Gudbjartsson DF. et al. 2010). Furthermore, a third SNP, rs13333226, located in the *UMOD* promoter within the previously mention LD block was described to be associated with higher predisposition to hypertension and cardiovascular disease in a large European case-control study (Padmanabhan S. et al. 2010). The common variant rs13333226 had the minor allele G associated with a protective effect for CKD and renal function. Further studies have reported a genetic association of *UMOD* promoter gene variants with end-stage renal disease, type 2 diabetic nephropathy, uric acid levels and increased risk of gout (Böger CA. et al. 2011; Ahluwalia TS. et al. 2011; Han J. et al. 2013). Only in the case of renal stones, *UMOD* SNPs held a protective role contrary to the rest of diseases. Köttgen et al. tried to find out the functional contribution of *UMOD* variants to the risk of developing CKD by conducting a case-control study of incident CKD. The study results indicated that *UMOD* variants exert a direct effect on UMOD urinary excretion that could cause progression of CKD and hypertension. Individuals carrying *UMOD* risk variants had significantly higher UMOD urinary levels in a dose-dependent manner (Köttgen A. et al. 2010). Adopting the GWA approach Olden et al. corroborate the role of *UMOD* variants in modulating UMOD excretion in

a large meta-analysis on more than 10000 individuals of European descent from three genetic isolates and three urban cohorts (Olden M. et al. 2014). The risk allele for CKD previously identified by Köttgen et al. was associated with higher UMOD levels and lower eGFR in the CKD Gen Consortium participants, in all six cohorts analyzed. The biological aspect of UMOD association has been disclosed in a recent work by Trudu et al. Using a transgenic mouse model that overexpresses UMOD (in comparison to the control) the authors documented an upregulation in *UMOD* gene expression when risk variants were present (*in vivo* and *in vitro* data). These results were evidenced at the protein levels in large cohorts. Over-expressing UMOD in *Umod* transgenic mice resulted in salt-sensitive hypertension suggesting a cause-effect relationship between *UMOD* risk variants and increased blood pressure (BP). This consequence is mainly triggered by the over-activation of the TAL NKCC2. This process is implicated in human hypertension, as BP was reduced after administering furosemide only in hypertensive participants homozygous for *UMOD* risk variants (Trudu M. et al. 2013). Graham et al.'s work also highlighted the role of UMOD in regulating BP by showing that UMOD deficient mice have lower baseline BP that is not increased by high-salt diet, in contrast to control mice (Graham LA. et al. 2014). This observation is coherent and corresponds to the one described in *Umod*-overexpressing mice, associating UMOD expression, salt intake and BP regulation.

Taken together, these studies contribute to understand the role of UMOD suggesting that this 'old' molecule retains several fundamental roles in the kidney and modifications affecting its levels and structure might cause common diseases as hypertension and CKD. Besides the variants in the *UMOD*, candidate-based analysis revealed some genes expressed in the TAL harbouring variants that might influence the excretion of uromodulin in the urine such as KCNJ1 (encoding ROMK), SORL1 and CAB39 proposing the existence of a regulatory networks that needs to be characterized through further investigation (Olden M. et al. 2014). More studies are needed to elucidate the role of UMOD in hypertension, CKD and possibly other common human diseases (e.g. UTI, nephrolithiasis) also to gain further insight into its complex biological functions and factors influencing its urinary excretion and interactions in the epithelial cells lining the TAL in the human kidney.

4. Urine osmolarity

Urine osmolarity is a useful biomarker that reflects the concentrating ability of the nephron, which itself plays a crucial role in regulating water and sodium excretion. Diluted urine is produced in case of increased water intake whereas urine more concentrated than blood is produced when water intake is decreased. In both cases, urinary sodium excretion and the total urinary solute excretion rate vary within narrow ranges. In contrast to solute excretion, urine osmolality varies widely in response to changes in water intake (Sands J. et al. 2009). Changes in urine osmolarity are affected by several factors of which the antidiuretic hormone vasopressin (ADH or AVP) that acts on the distal convoluted tubule and the principal cells of the collecting duct. In addition to its association with water disorders, it has been shown that AVP is significantly associated with the incidence or progression of diseases such CKD, ADPKD, diabetic nephropathy, obesity, metabolic syndrome, and insulin resistance (Devuyst O. et al. 2013; Bardoux P. et al. 2013; Enhorning S. et al. 2011; Roussel R. et al. 2011; Ho TA. et al. 2012; Ponte B. et al. 2015). Therefore, studying AVP and its effects has become of significant importance. However, measuring circulating levels of AVP is challenging because of its small mass, very low circulating concentrations, poor stability in vitro, and time-consuming assays. For this purpose, the majority of current studies use copeptin (surrogate of AVP) or urine osmolarity, which reflects the action of AVP on distal tubular segments of the kidney (Clark WF. et al. 2011; Strippoli GF. et al. 2011). Considering the technical constraints in measuring urine osmolarity, surrogates of this parameter have been suggested using different equations that are based on the urinary levels of sodium and other components that are easily measurable. These surrogates lack validation in in the general population.

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II. Aim of the thesis

Despite its discovery more than 50 years ago, little is known about the function of uromodulin, the most abundant protein in normal human urine.

The aim of this thesis was (1) to develop and characterize an assay to accurately measure the levels of uromodulin in the urine and to use this assay to (2) investigate the effect of a common variant in the promoter region of *UMOD* gene on the biochemistry of this glycoprotein and its circulating levels. We (3) employed our assay to determine the clinical and biological factors influencing the levels of uromodulin in urine.

In the first part of this thesis, we developed an ELISA to measure uromodulin in human urine. We used this assay to explore the stability of uromodulin under different treatments and storage conditions. We then characterized the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in the urine in large cohorts.

In the second part of the thesis, we explored the potential impact of a common promoter variant of *UMOD* on urine and plasma level and on biochemical properties of uromodulin by measuring uromodulin levels in two Swiss urban cohorts.

In the third part, we studied the influence of clinical, biological and genetic factors on the uromodulin levels, based on several large cohorts.

In the final part, we validated two formulas as surrogates for urine osmolarity, an integrative biomarker of kidney function, in the general population as well as in chronic kidney disease patients.

Taken together, our results presented a reliable tool and consistent procedure allowing an accurate measurement of uromodulin in the urine. We also evidenced factors that affect the biochemistry and the excretion levels of uromodulin in urine and plasma. Finally, we validated uromodulin as a biomarker for tubular mass and function in the general population.

III. Determination of Uromodulin in Human Urine: Influence of Storage and Processing

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Abstract

Uromodulin (Tamm-Horsfall protein) is the most abundant protein excreted in the urine under physiological conditions. It is exclusively produced in the kidney and secreted into the urine via proteolytic cleavage. The involvement of UMOD, the gene that encodes uromodulin, in rare autosomal dominant diseases, and its robust genome-wide association with the risk of chronic kidney disease suggest that the level of uromodulin in urine could represent a critical biomarker for kidney function. The structure of uromodulin is complex, with multiple disulfide bonds and typical domains of extracellular proteins. Thus far, the conditions influencing stability and measurement of uromodulin in human urine have not been systematically investigated, giving inconsistent results. In this study, we used a robust, in-house ELISA to characterize the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in the urine. The levels of uromodulin in human urine were significantly affected by centrifugation and vortexing, as well as by the conditions and duration of storage. These results validate a simple, low-cost ELISA and document the optimal conditions of processing and storage for measuring uromodulin in human urine.

Introduction

Urinary biomarkers constitute an essential tool for the diagnosis, classification, and prognosis of kidney diseases (Koyner JL. et al. 2010). Recent evidence pointed at uromodulin (originally named Tamm-Horsfall glycoprotein) as a potential urinary biomarker relevant for renal function, chronic kidney disease (CKD) and hypertension (Tamm I. et al. 1950; Rampoldi L. et al. 2011). Uromodulin is a 105 kD glycoprotein with seven N-glycosylation sites and a high-mannose chain. The protein contains 616 amino acids including 48 cysteine residues that are all engaged in the formation of disulfide bonds. Uromodulin contains three epidermal growth factor (EGF)-like domains and a zona pellucida (ZP) domain, found in many extracellular proteins, as well as a glycosylphosphatidylinositol (GPI)-anchoring site (Rampoldi L. et al. 2011). Uromodulin is a kidney-specific protein that is exclusively synthesized in the epithelial cells lining the thick ascending limb (TAL) of Henle's loop (Chabardès-Garonne D. et al. 2003). After proper trafficking and maturation in TAL-lining cells, uromodulin reaches the apical plasma membrane, to be cleaved and assembled in the urine as polymers forming a gel-like structure (Wiggins RC. et al. 1987).

Uromodulin is produced at very high rate in the TAL, and is by far the most abundant protein in normal urine (excretion: 50-100 mg/day) (Serafini-Cessi F. et al. 2003). Functions attributed to uromodulin include protection against urinary tract infections; prevention of renal calculi formation by reducing aggregation of calcium crystals and influencing transport processes by regulating the activity of NKCC2 and/or ROMK (Renigunta A. et al. 2011; Mutig K. et al. 2011). Interest for uromodulin was re-ignited when it was discovered that mutations in the UMOD gene that codes for uromodulin are responsible for a series of monogenic disorders (familial juvenile hyperuricemic nephropathy, medullary cystic kidney disease type 2 or glomerulocystic kidney disease) all known as uromodulin-associated kidney disease (UAKD) (Rampoldi L. et al. 2011). These disorders are characterized by severe tubulointerstitial damage, defective urinary concentration, hyperuricemia and gout, and progressive renal failure (Bollée G. et al. 2011). The mutations often affect cysteine residues, resulting in conformational changes and intracellular aggregates of uromodulin. In turn, there is a strong decrease in the secretion of the protein by the TAL cells and a strong decrease in the urinary excretion of uromodulin (Dahan K. et al. 2003; Bleyer AJ. et al. 2004; Bernascone I. et al. 2010). Lately, a number of genome wide association studies (GWAS) revealed that variants in the UMOD gene were strongly

associated with markers of renal function and risk of developing hypertension and CKD in the general population (Padmanabhan S. et al. 2010; Köttgen A. et al. 2009; Chambers JC. et al. 2010). The association of uromodulin with both monogenic diseases and complex disorders such as CKD and hypertension provides a strong rationale for evaluating its urinary concentration as a biomarker for renal function and CKD.

The determination of uromodulin in the urine is hampered primarily by its capacity to aggregate and the potential instability of such a complex protein. Documentation of sampling, processing and storage conditions is thus crucial for accurate uromodulin quantification. Despite the early characterization of antibodies specific for human uromodulin (Brunisholz M. et al. 1986), the few reports on uromodulin dosage yielded conflicting results in terms of stability, storage conditions and processing of human urine (Akesson I. et al. 1978; Uto I. et al. 1992; Torffvit O. et al. 1992; Kobayashi K. et al. 2001). Important points such as the potential influence of urine centrifugation or vortexing, acidification or alkalinization, treatment with protease inhibitors or normalization for urinary creatinine remain unsolved. Furthermore, earlier immunoassays were often based on poorly documented anti-uromodulin antibodies. Considering the increasing interest for a robust determination of uromodulin in the urine, the need for a high-throughput assay and the limited and contradictory information available, we developed and characterized a robust ELISA for uromodulin and used this assay to investigate the stability of uromodulin under different treatment and storage conditions of human urine.

Material and Methods

Urine sample collection, storage and handling

Analyses were performed on second morning urine samples collected (mid-stream) in a sterile container from healthy volunteers aged 18-50 years, and processed within 2 h. This protocol was approved by the Ethical Committee of the Université catholique de Louvain.

The influence of human urine sample processing on the determination of uromodulin (Figure 1) was tested after vortexing the sample for 10 sec (Vortex-Genie 2, FAUST, Schaffhausen, Switzerland); centrifugation for 10 min at 3,600 rpm (Eppendorf Centrifuge 5430, Hamburg, Germany) at room temperature (i.e. standard protocol for urine processing and removing cells and debris; Thomas CE. et al. 2010); treatment with protease inhibitors (Leupeptin 1 µmol/L; Sigma-Aldrich, St. Gallen, Switzerland; sodium azide 10 mmol/L); pH

adjustment performed by drop titration with 1 N HCl (to pH 2.0) or with 1 N NaOH (to pH 8.0) using a Hanna HI 2211 pH meter; dilution using ultrapure deionized water (Destamat Bi 18E, QCS, Maintal, Germany) vs. TEA buffer (0.5 % Triton X-100, 20 mM EDTA, pH 7.5).

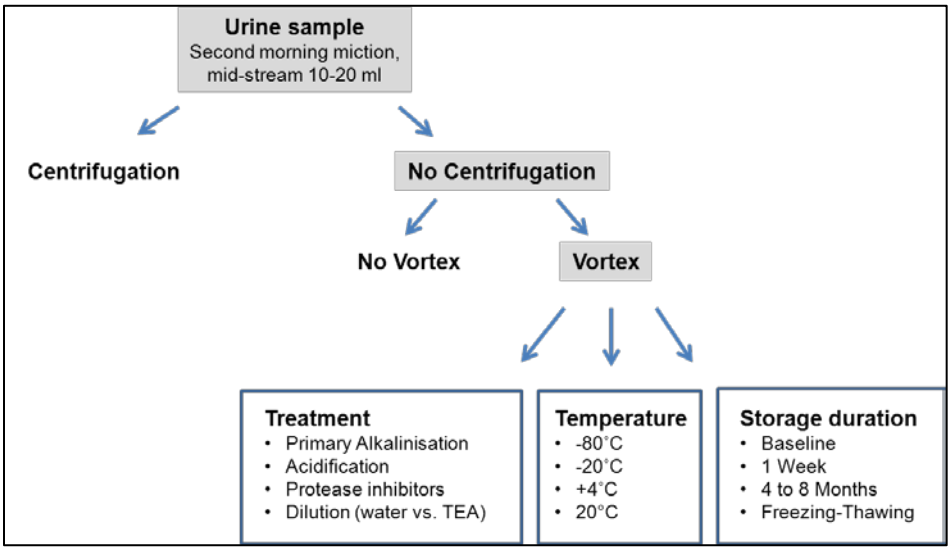

Figure 1: Processing of urine samples for uromodulin determination. The flow chart describes how urine samples were collected and treated to validate the protocol of uromodulin determination. Grey boxes represent the standard treatment to measure uromodulin in the urine

The effect of storage conditions was tested by comparing baseline levels with 1-week and 5-month storage at room temperature, +4 °C and -20 °C; 4-month and 8-month storage at -80 °C; five cycles of freezing-thawing (sample kept at -80 °C for 48 h followed by thawing on ice). Different sample sets were used to evaluate the influence of the various processing conditions as described.

Uromodulin ELISA

The in-house ELISA for uromodulin is a colorimetric based sandwich immunoassay using a sheep anti-human uromodulin antibody (Meridian Life Science, Memphis, USA; K90071C) as the capture antibody. This antibody gives a single arc when tested by immuno-electrophoresis against fresh urine. The primary antibody was a monoclonal anti-human uromodulin antibody (Cedarlane Laboratories, Burlington, USA; CL 1032A) raised in mouse and validated in solid phase radioimmunoassay. The secondary antibody was a goat anti-mouse IgG (H+L) horseradish

peroxidase conjugated (Bio-Rad, Cressier, Switzerland; 172.1011). The substrate was O-Phenylenediamine dihydrochloride (OPD) (10 mg/tablet) (Sigma-Aldrich). The OPD substrate solution was freshly prepared by dissolving a tablet in 25 mL of phosphate-citrate buffer (0.1 M citric acid monohydrate, 0.2 M Na_2HPO_4), pH 5.5. A volume of 5 µL 30 % H_2O_2 was added to 25 mL of substrate solution. Human uromodulin (Millipore, Billerica, USA) was used to establish the standard curve, with freshly prepared serial dilutions from the standard stock solution (100 µg/mL). Both the standard curve and a standard sample (uromodulin concentration 25 µg/mL) were systematically used for quality control (QC). The determination of urinary uromodulin by ELISA was carried out as follows: a 96-well microtiter plate (NUNC MaxiSorp™, eBioscience, Vienna, Austria) was coated with 100 µL of 5 µg/mL capture antibody in coating buffer (500 mM H_3BO_3, 500 mM KCl, 345 mM NaOH, pH 9.0). The plate covered with adhesive seal was incubated at 4 °C overnight then washed three times with freshly prepared washing buffer (0.1 % Tween 20 in 10 mM phosphate buffer saline (PBS) pH 7.2 (PBS-Tween 0.1 %)) using ImmunoWash 1575 Microplate Washer (Bio-Rad). Unoccupied sites on the plate were blocked with 100 µL blocking buffer (0.5 % BSA in 10 mM PBS, pH 7.2) and incubated at 37 °C for 1 h with a slow rotation (100 rpm). The plate was then washed three times with washing buffer and placed upside-down on absorbent paper to remove residual buffer. 100 mL of PBS-Tween 0.1 % was dispensed in all wells. Urine samples were stabilized at room temperature then diluted 1:50 in ultrapure deionized water, as preliminary testing revealed no significant difference vs. dilution with TEA buffer (data not shown). A volume of 100 µL per well was distributed into the coated wells after vortexing. Standards and QC sample were run in duplicate whereas each urine sample was tested in 3 different dilutions. Deionized water was used as blank. After 1 h incubation at 37 °C, the plate was washed three times and placed on absorbent paper. 100 µL of primary antibody diluted in PBS-Tween 0.1 % (1 µg/mL) was dispensed in each well; the plate was incubated at 37 °C for 1 h (rotation, 100 rpm) then washed three times. The secondary antibody diluted 1: 2,000 in PBS-Tween 0.1 % was added to the wells for 45 min at 37 °C and the plate washed three times. Colour was developed by adding 100 µL of OPD substrate solution. The plate was incubated at room temperature in the dark for 1 min, and the reaction stopped by adding 50 µL of 2 M H_2SO_4 to each well. Optical density (Infinite M200Pro, Tecan; Grödig, Austria) was read at 492 nm and urinary uromodulin concentration was determined by referring to the standard curve. Uromodulin levels obtained

using the in-house ELISA were compared to the commercial ELISA from MD Bioproduct (St. Paul, USA; M036020), following the protocol given by the manufacturer. This test has been used in several studies (Padmanabhan S. et al. 2010, Reznichenko A. et al. 2011). Urinary creatinine levels (normalization) were measured using the Synchron® System Creatinine Assay (Unicell DxC Synchron®, Beckman Coulter, Brea, USA), following the manufacturer's instructions.

Immunoblotting

Kidneys from Umod mice (Mo L. et al. 2004) were grounded in liquid nitrogen and homogenized as described previously (Bernascone I. et al. 2010). The homogenate was centrifuged at 1000 × g for 15 min at 4 °C and the resulting supernatant at 100,000 × g for 120 min at 4 °C. The pellet was suspended in homogenization buffer before determination of protein concentration (Pierce BCA protein assay kit; Thermo Fischer Scientific; Rockford, USA). SDS-PAGE for mouse and human samples was performed under reducing conditions. Samples (20 µg of mouse and human kidney extract; 2 µL of urine) were loaded after being mixed with Laemmli sample buffer and heated for 5 min at 95 °C (kidney samples). Proteins were separated on 10 % SDS gel and transferred to nitrocellulose membrane for Western blotting. Membranes were blocked with 5 % milk blot for 30 min at room temperature then incubated overnight at 4 °C with either sheep or mouse anti-uromodulin antibodies (1:400 in 0.5 % BSA blocking buffer). Secondary antibodies were goat anti-mouse HPR conjugated (1:10,000) or polyclonal rabbit anti-sheep HRP conjugated (1:1,000), for 1 h at room temperature. Antigen-antibody reaction was detected by using ECL (Immun-Star HRP, Bio-Rad) and light-sensitive film (GE Healthcare, Glattbrugg, Switzerland). The molecular weight was estimated by running the Precision Plus Protein™ All Blue standard (Bio-Rad).

Deglycosylation and desialylation of uromodulin

Deglycosylation of uromodulin from human urine was carried out using PNGase F (PNGase F P0704S, New England Biolabs, Ipswich, USA) following the manufacturer's protocol, whereas desialylation was performed according to the protocol described by Parsons et al. (Parsons CL. et al. 2007). Briefly, uromodulin was precipitated from pooled human urine (1.5 L) following the protocol of Tamm and Horsfall (Tamm I. et al. 1950), dialyzed overnight at 4 ˚C and then lyophilized (Virtis, Kloten Switzerland). Dry uromodulin was solubilized in 2.5 M acetic acid (10 mg/ml), heated for 3 h at 82 °C, and then washed 3 times with 15 mL PBS (pH 7.2) on

Centricon (MWCO 30000) cartridge (Millipore). 1.5 µL of deglycosylated urine and 0.1 µL of desialylated uromodulin (vs. 0.5 L of untreated urine) were loaded on 10 % acrylamide gel and analyzed as described above.

Immunohistochemistry

Colocalization of uromodulin with NKCC2 was carried out in cryosections of human and mouse kidney samples as previously described (Dahan K. et al. 2010; Bernascone I. et al. 2003). Briefly, 5 µm-thick cryosections were blocked with 1 % BSA, 0.02 % sodium azide-PBS for 30 min at room temperature, incubated for 2 h at room temperature with the sheep (1:400) or mouse (1:200) antibodies against human uromodulin, followed by washing and incubation with AlexaFluor633-conjugated donkey anti-sheep or goat anti-mouse (1:200) for 90 min at room temperature. Uromodulin-stained sections were then incubated with a polyclonal rabbit anti-NKCC2 antibody (Millipore; AB3562P; 1:100) for 3 h at room temperature, followed by washing and incubation with Alexafluor488-conjugated goat anti-rabbit antibodies (1:200). Sections were viewed on a Leica SP5 confocal microscope.

Surface plasmon resonance: Biacore

The interaction between uromodulin and the capture antibody was analyzed by surface plasmon resonance, using a Biacore T100 system (GE Healthcare, Uppsala, Sweden). Chemicals were from Sigma unless otherwise noticed. Binding experiments were performed in PBS buffer pH 7.4 containing 0.2 % of Tween 20 at a flow rate of 30 µL/min at 25 °C. Ultrapure and filtered water (“MilliQ”, Millipore, Billerica, USA) was used for preparing all solutions. The carboxymethyl dextran chip (CMD500L, XanTec bioanalytics, Düsseldorf, Germany) surface (1.2 mm^2 area) was cleaned before use by injecting 7 times a 50 mM NaOH solution containing 1 M NaCl for 30 sec at a flow rate of 5 µL/min. Surface binding is expressed in terms of changes in response units (RU) with 1 RU being approximately 1 pg/mm^2. Sheep polyclonal anti-uromodulin antibody (300 nM) in PBS-Tween was immobilized by amine coupling to the chip surface activated with aqueous solutions of 0.4 M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 0.1 M N-hydroxysuccinimide for 300 sec at 5 µL/min flow rate. For determination of kinetic constants, a dilution series of four concentrations (19 nM, 39 nM, 78 nM, 156 nM) of uromodulin was injected using the T100 in multichannel mode. The reference channel used in parallel did not contain immobilized antibody, in order to detect background response and unspecific binding of analyte to the surface. Between two measurements, the

surface was regenerated by injecting twice 10 mM glycine at pH 2 for 30 sec, which completely removed uromodulin from the antibody. For data evaluation, the measured sensorgrams were referenced twice, first by subtracting the signal from the reference channel, and second by subtracting the signal obtained from injected pure buffer solution. Kinetic curves were evaluated using Biacore T100 Evaluation Software (v. 2.0.2). A global fit was performed using the entire concentration series. Rate constants for association and dissociation were calculated by taking a 1:1 binding model as a basis.

Data analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 19 (IBM Corp., Armonk, USA). The Pearson correlation coefficient was used for correlation analysis, whereas ANOVA and paired t-test were used for comparisons between the groups. A Bland-Altman plot was used to evaluate agreement between uromodulin levels measured with the in-house ELISA and commercial kit. Level of significance was set to $p < 0.05$.

Results

Characterization of the antibodies against human uromodulin

The antibodies used for the in-house ELISA were characterized by immunoblotting and immunostaining (Figure 2). Immunoblot analysis of human urine and kidney samples in parallel with mouse kidney samples using the sheep polyclonal antibodies detected the uromodulin band at ~100 kDa in all samples except the Umod KO kidney sample. The uromodulin band was also detected in human urine and kidney samples using the mouse monoclonal antibody (Figure 2A, top panel).

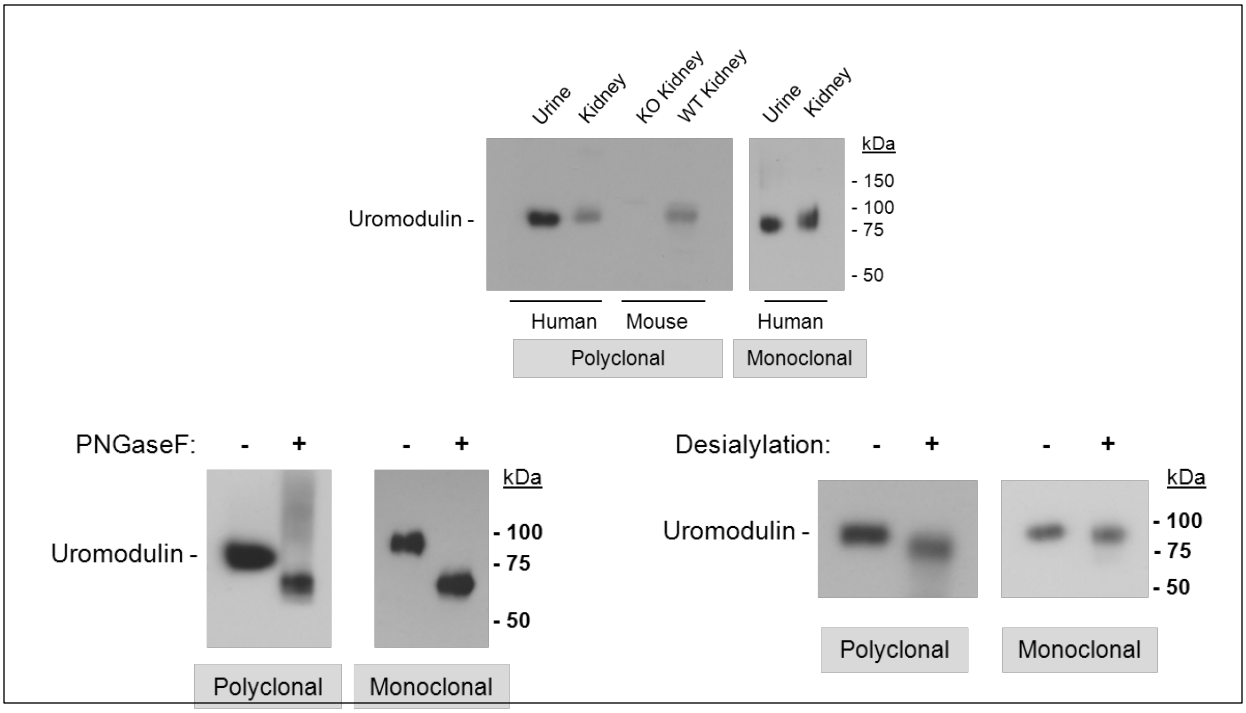

Figure 2A (Top panel): Characterization of anti-human uromodulin antibodies. Western blot analysis (10 % acrylamide gel) of human urine (2 µL), human kidney tissue (20 µg), and Umod knock-out (KO) and wild-type (WT) mouse kidney tissue (20 µg) using the sheep polyclonal or the mouse monoclonal antibodies against human uromodulin. A single band at ~100 kDa is detected with both antibodies, and absent in the Umod KO mouse kidney. Figure 2A (Lower panel): Characterization of anti-human uromodulin antibodies. The changes in molecular mass resulting from deglycosylation (left panel) and desialylation (right panel) of uromodulin are evidenced by using both the polyclonal and monoclonal anti-uromodulin antibodies. 1.5 µL of deglycosylated urine and 0.1 µL of desialylated uromodulin (vs. 0.5 L of untreated urine) were loaded on the gel.

Both the polyclonal and monoclonal antibodies also appropriately identified the deglycosylated and desialylated forms of uromodulin (Figure 2A, lower panels).

Staining of human and mouse kidney tissue samples with the mouse monoclonal or the sheep polyclonal antibodies detected uromodulin in the TAL, where it colocalized with NKCC2 at the apical surface area (Figure 2B).

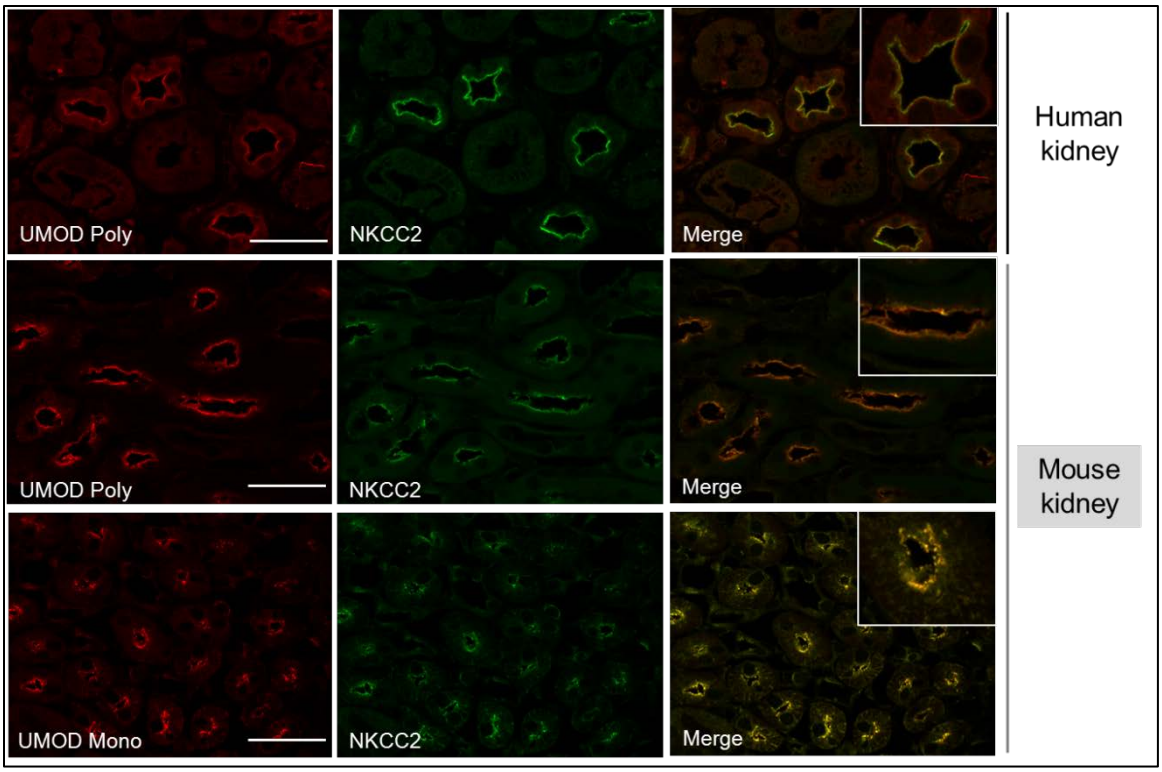

Figure 2B: Characterization of anti-human uromodulin antibodies. Immunostaining of human cortical kidney sections (top row) using polyclonal sheep antibodies against human uromodulin (red), evidencing the apical staining in thick ascending limb profiles that are also positive for NKCC2 (green). A similar co-distribution between uromodulin (red) and NKCC2 (green) is observed in mouse kidney, using the polyclonal (middle row) or monoclonal (bottom row) anti-uromodulin antibodies. Scale bar: 10 µm, inset: 2x zoom.

Surface plasmon resonance (van der Merwe PA. 2001) was further used to characterize the uromodulin-antibody interaction (Figure 2C).

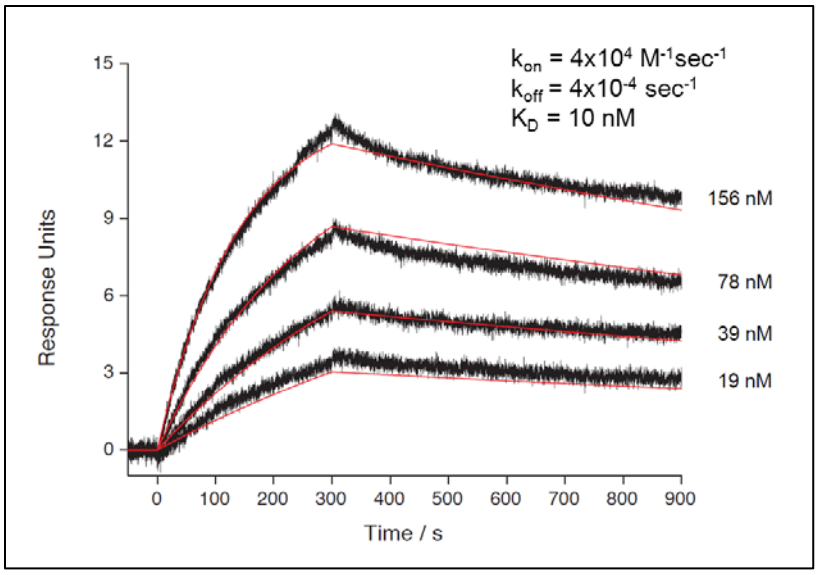

Figure 2C: Characterization of anti-human uromodulin antibodies. Sensorgrams for the interaction of purified human uromodulin (19 - 156 nM) with the immobilized sheep anti-uromodulin antibody by surface plasmon resonance technique using the Biacore system. Uromodulin was injected at 0 for 300 sec. Red lines are the result of a global fit. The constant of dissociation (KD) was determined after evaluating the association (k_{on}) and dissociation (k_{off}) rate constants simultaneously using 1:1 kinetic binding model.

As the isoelectric point of uromodulin (pI = 3.2) is too low for its immobilization to a carboxymethyl dextran surface, the capture sheep anti-uromodulin antibodies were immobilized at the surface of a sensor chip, and a dilution series of uromodulin was injected. For the interaction of immobilized sheep anti-uromodulin antibody with uromodulin, rate constants for association (k_{on}) and dissociation (k_{off}) of 4 x 104 M^{-1} s^{-1} and 4 x 10^{-4} s^{-1}, respectively, were determined, giving a dissociation constant KD (= k_{off}/k_{on}) of 10 nM. We also measured a strong binding response for the interaction of mouse anti-human uromodulin antibody to the sheep anti-uromodulin antibody - uromodulin complex. This situation is comparable to the conditions in ELISA (see below). Regeneration of the surface with 10 mM glycine at pH 2.0 removed both the antibody and uromodulin.

Kit	Detection range (standard curve)	Inter-assay Variability	Intra-assay Variability
In-house	3.9 - 500 ng/mL	3.28 %	5.46 %
MD Bioproduct (Cat. M036020)	2.34 - 150 ng/mL	11.63 %	8.36 %
BioVendor (Cat. RD191163200R)	0.5 - 32 ng/mL	6.4 %	2 %
USCN Life Science Inc. (Cat. E96918 Hu)	3.13 - 200 ng/mL	< 12 %	< 10 %

Table 1: Comparison of the characteristics of the in-house ELISA for uromodulin and the available commercial ELISA kits.

Characteristics of the ELISA for uromodulin

When tested against purified human uromodulin, the in-house ELISA for human uromodulin showed a sensitivity (minimum amount of analyte which can be accurately detected) of 2.8 ng/mL and a linearity (correlation between concentration and optical density) of 1.0 (Figure 3A).

The inter- and intra-assay variabilities were determined at 3.28 % and 5.46 %, respectively. The assay had a detection range between 3.9 and 500 ng/mL. When compared with other assays, the in-house ELISA showed a wider range of measurement and lower intra- and inter-assay variability than commercially available routine kits (Table 1).

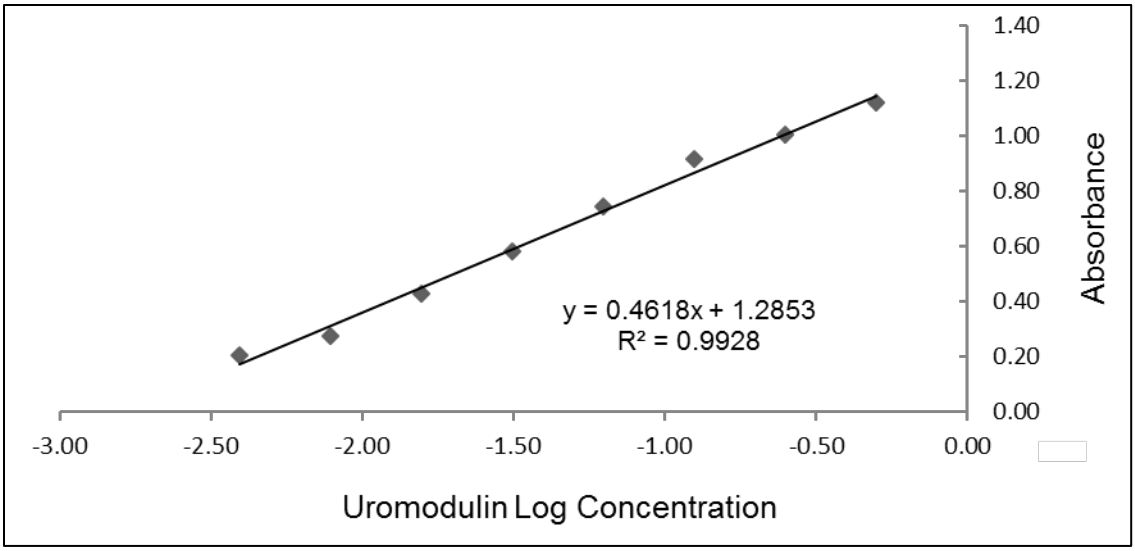

Figure 3A: Characterization of the in-house ELISA for uromodulin. Standard curve of absorbance for a dilution series (500, 250, 125, 68, 34, 17, 8.5 and 3.9 ng/mL) of purified human uromodulin.

There was a robust correlation ($r = 0.905$, $p < 0.001$) when comparing the in-house ELISA with the MD Bioproduct kit. The Bland-Altman plot showed that the mean difference between both methods was -1.47 µg/mL (95 % CI, -3.21 to 0.27 µg/mL) (Figure 3B).

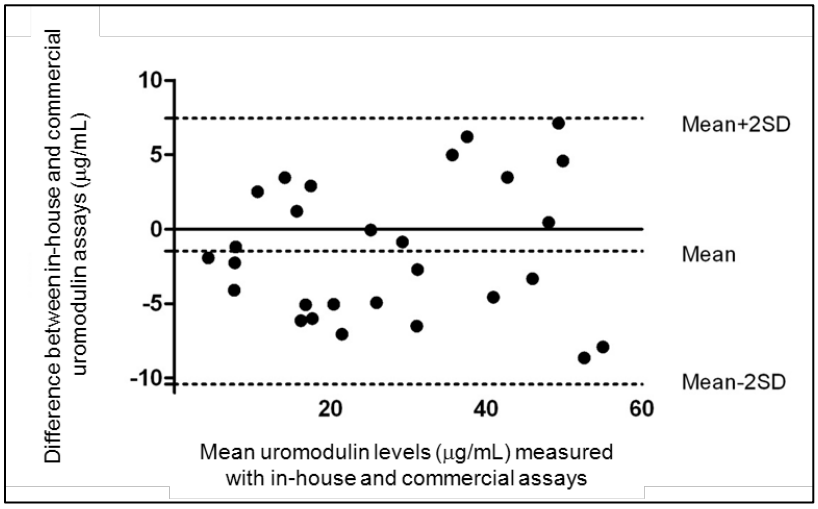

Figure 3B: Characterization of the in-house ELISA for uromodulin. Bland-Altman plot: The difference between uromodulin levels measured with in-house ELISA and the commercial kit plotted against mean uromodulin levels measured with both methods (n = 28). Horizontal lines represent the mean difference for the whole group (-1.47 g/mL) and the 95 % limits of agreement (-10.40 g/mL to 7.45 g/mL).

Influence of processing of urine samples

Since uromodulin has a tendency to aggregate, we first investigated the potential influence of vortexing and centrifugation on the determination of uromodulin levels in human urine (Table 2).

	Unindexed Uromodulin (µg/mL)	**P**	**Indexed Uromodulin** (mg/gr creat)	**P**	**N**
Vortex	11.03 ± 1.67	0.001	15.90 ± 1.45	0.001	37
No vortex	5.02 ± 0.66		10.84 ± 0.54		
Centrifugation	6.40 ± 0.63	< 0.001	9.97 ± 1.43	< 0.001	53
No centrifugation	13.27 ± 1.18		15.66 ± 1.34		

Table 2: Effect of sample processing (vortex, centrifugation) on the concentration of uromodulin in the urine. Urine samples were vortexed for 10 s. Centrifugation was performed for 10 min at 3600 rpm at room temperature. Two different sets of samples were used to test the influence of vortexing (N = 37) and centrifugation (N = 53).

Comparison of fresh samples assayed before and after vortexing revealed a more than 50 % increase in uromodulin levels (unindexed uromodulin: 5.02 ± 0.66 vs. 11.03 ± 1.67 g/mL, respectively, $p = 0.001$; indexed uromodulin: 10.84 ± 0.54 vs. 15.90 ± 1.45 mg/gr creat, respectively, $p = 0.001$, $n = 37$). Treating the urine samples with an usual centrifugation protocol (10 min, 3600 rpm) also showed a strong effect, since centrifugation yielded a significant decrease in unindexed (6.40 ± 0.63 vs. 13.27 ± 1.18 g/mL, respectively, $p < 0.001$) and indexed (9.97 ± 1.43 vs. 15.66 ± 1.34 mg/gr creat, respectively, $p < 0.001$, $n = 53$) uromodulin levels. Immunoblotting analyses (Figure 4) revealed that centrifugation was responsible for the precipitation of uromodulin in the pellet of cell debris.

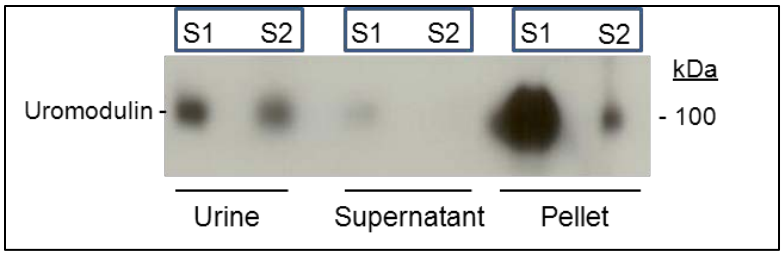

Figure 4: Effect of urine centrifugation on the detection of uromodulin. Western blot analysis (10 % acrylamide gel) of two human urine samples (S1, S2) using the polyclonal sheep anti-uromodulin antibody. The signal obtained in baseline urine is lost when analyzing the supernatant following centrifugation, whereas a clear signal appears in the pellet. Similar volumes (2 L) of untreated urine, supernatant and re suspended pellet were loaded.

In comparison with the uromodulin band detected in fresh, non-centrifuged urine samples, the signal was strongly attenuated in the centrifuged urine sample while becoming apparent in the resulting pellet.

Alkalinization of fresh urine sample to pH 8.0 did not influence the determination of urinary uromodulin, as compared with untreated (mean pH 5.68 ± 0.19) samples (unindexed uromodulin: 19.25 ± 4.14 vs. 20.60 ± 5.24 g/mL, respectively, p = 0.179; indexed uromodulin: 17.56 ± 2.28 vs. 18.34 ± 2.67 mg/g creat, respectively, p = 0.260, n = 14). Likewise, acidification of urine samples to pH 2.0 did not result in a significant difference between values from untreated (mean pH 6.15 ± 0.59) samples (unindexed uromodulin: 10.01 ± 2.25 vs. 9.73 ± 2.07 µg/mL, respectively, p = 0.621; indexed uromodulin: 18.86 ± 8.26 vs. 19.10 ± 8.55 mg/g creat, respectively, p = 0.782, n = 8).

Influence of storage conditions

In order to cast light on the influence of storage conditions on the stability of uromodulin, we compared values obtained in samples analyzed at baseline and after 1 week or 5 months storage at room temperature, +4 °C and -20 °C. As compared to baseline, storage at either room temperature or 4 °C or even -20 °C were associated with decreased levels of both unindexed and indexed uromodulin (Table 3).

		Unindexed Uromodulin (µg/mL)	P	Indexed Uromodulin (mg/g creat)	P	N
1 week storage	**Baseline**	12.39 ± 2.41		22.70 ± 3.35		
	RT	6.14 ± 1.34*		13.00 ± 2.34*		
	+4 °C	7.22 ± 1.60*	0.078#	11.49 ± 1.50*	0.014#	13
	-20 °C	9.98 ± 1.96*		18.69 ± 3.18*		
4 month storage	**Baseline**	36.37 ± 2.62	0.354	24.05 ± 1.26	0.412	61
	-80 °C	35.47 ± 2.32		23.30 ± 1.25		
5 month storage	**Baseline**	28.50 ± 6.76		26.48 ± 3.45		
	+4 °C	10.17 ± 3.96*		10.27 ± 2.35*		
	-20 °C	16.52 ± 5.08*	0.068#	15.78 ± 2.73*	0.001#	10
	+4 °C & PI	11.04 ± 4.69*		10.80 ± 2.32*		
	-20 °C & PI	20.23 ± 5.27*£	0.111#	18.71 ± 2.69*£	0.003#	

Table 3: Effect of storage conditions (duration, temperature, protease inhibitors) on the concentration of uromodulin in the urine. PI, treatment with protease inhibitors (Leupeptin and sodium azide). Three different sets of urine samples were used to assess influence of storage after 1 week (N = 13), 4 months (N = 61) and 5 months (N = 10) vs. baseline levels. * $p < 0.05$ storage condition vs. baseline, £ $p < 0.05$ no vs. protease inhibitors, paired t tests; # ANOVA.

Addition of protease inhibitors at the time of collection had some effect on the degradation of the samples conserved at -20 °C, but not on those kept at +4 °C. In any case, the addition of protease inhibitors was insufficient to prevent a significant decrease in the uromodulin levels as compared to baseline values. In contrast, 4-month storage at -80 °C was not associated with significant changes in uromodulin levels in untreated samples. Further analyses revealed a slight but significant decrease after 8 months storage at -80 °C (baseline uromodulin: 23.73 ± 1.57 µg/mL vs. 8-month: 20.13 ± 1.17 µg/mL, $p = 0.023$, $n = 142$). Freezing-thawing cycles (from -80 °C to 0 °C) showed no significant changes in the levels of urinary uromodulin as compared to baseline (Table 4).

		Unindexed Uromodulin (µg/mL)	**P**	**Indexed Uromodulin** (mg/gr creat)	**P**	**N**
Freezing-thawing cycles (-80 °C to 0 °C)	**Baseline**	22.96 ± 4.75	0.616#	18.14 ± 2.82	0.351#	8
	1	22.01 ± 3.81		20.19 ± 3.51		
	2	24.74 ± 4.05		23.67 ± 3.07		
	3	16.74 ± 2.68		18.17 ± 2.58		
	4	16.96 ± 3.36		16.20 ± 1.98		
	5	19.42 ± 4.56		15.91 ± 2.42		

Table 4**:** Effect of freezing-thawing cycles on the concentration of uromodulin in the urine.
ANOVA

Discussion

Increasing evidence suggests that the level of uromodulin in urine could represent a useful biomarker for kidney function (Rampoldi L. et al. 2011; Köttgen A. et al. 2010). In this study, we validated an efficient and cost-effective immunoassay and characterized the conditions of sampling and storage necessary to provide a faithful dosage of uromodulin in human urine. The urinary uromodulin levels were significantly affected by centrifugation and vortexing, as well as by the conditions and duration of storage.

To develop our in-house ELISA we used commercially available anti-uromodulin antibodies and validated their specificity in human and mouse kidney and urine samples. Both antibodies evidenced the ~100 kDa band corresponding to uromodulin on Western blot, either in native or deglycosylated/desialylated state. They also showed the typical distribution along with NKCC2 in the apical membrane of the TAL. We used plasmon surface resonance to determine the binding constant for interaction of the immobilized sheep anti-uromodulin antibody to uromodulin to 10 nM which is in the expected range for an antibody-protein interaction. The immunoassay standard curve showed linearity over a broad range of values, allowing the detection of uromodulin with high sensitivity and very low inter- and intra-assay variability. It must be noted that, in contrast with previous results based on immunoblotting (Kobayashi K. et al. 2001), dilution of the samples with deionized water yielded similar results as with TEA buffer. All these features, combined with an excellent correlation with the most used commercial

ELISA, substantiate the interest of our immunoassay with the advantage of low cost, wide range of detection, and low variability.

Our analyses revealed a striking effect of vortexing and centrifugation on the determination of uromodulin in the urine. These two procedures yielded variations reaching 50 % of the levels obtained on control, unprocessed samples (Table 2). These findings are clinically relevant, because low levels of urinary uromodulin have been suggested to be of diagnostic value in UAKD (Rampoldi L. et al. 2011; Bollée G. et al. 2011; Dahan K. et al. 2003; Bleyer AJ. et al. 2004). The effect of vortexing confirms the importance of the aggregation of uromodulin molecules in the normal urine. Uromodulin is also known to cofractionate with exosomes (Hiemstra TF. et al. 2011), the recovery of which is increased by vortexing (Zhou H. et al. 2011). Uto et al. previously suggested that uromodulin may be trapped in cell debris or aggregated with crystals (Uto I. et al. 1991) after centrifugation protocols that are usual to remove contamination due to lysis or suspended cells (Waikar SS. et al. 2010). Our data confirm these findings and show that centrifugation of urine may decrease the level of uromodulin by ~30 %. Thawed urine samples should thus be vortexed but not centrifuged before assaying uromodulin.

The question of the stability of uromodulin during different storage protocols is critical for analyzing large, multicentric cohorts. Previous studies based on small sample size yielded inconsistent conclusions about the influence of storage duration and temperature (Akesson I. et al. 1978; Uto I. et al. 1991; Torffvit O. et al. 1992; Kobayashi K. et al. 2001). Furthermore, these studies did not take into account normalization for urinary creatinine, which is usual for kidney biomarkers – at least in a stable situation (Waikar SS. et al. 2010; Ortiz A. et al. 2011). Our results, obtained on a large number of samples, reveal that short (1 week) and longer (5 months) storage at room temperature, 4 °C or -20 °C causes a significant decrease in indexed urinary uromodulin levels, largely due to decreased uromodulin. In contrast, 4-month storage at -80 °C is associated with marginal, non-significant decrease in the unindexed and indexed values. Of note, the decrease in unindexed uromodulin levels becomes significant after a 8-month storage at -80 °C. The fact that storage of untreated urine samples at room temperature, 4 °C or -20 °C significantly decreases the level of uromodulin substantiates the observations of Kobayashi et al (Kobayashi K. et al. 2001). This effect is only partially attenuated with protease inhibitors, which show some effect for samples kept at -20 °C - but insufficient to prevent a significant degradation. Taken together, these data confirm the fact that urine samples should be stored at

-80 °C and analyzed within 3 months to give the most reliable measurements. Of note, up to 5 freezing-thawing cycles on ice did not affect the stability of uromodulin stored at -80 °C.

Previous studies also reported inconsistent results in terms of treatments (detergents or TEA buffer, alkalinization) supposed to solubilize aggregates of uromodulin in urine (Akesson I. et al. 1978; Uto I. et al. 1991; Torffvit O. et al. 1992; Kobayashi K. et al. 2001; Dawney AB. et al. 1982). Some of these treatments may interfere with the binding of uromodulin to the ELISA capture antibody (Uto I. et al. 1991). We verified here that dilution with deionized water gave similar results than with TEA, and that urine alkalinization (or acidification) had no effect on the determination of uromodulin. These data support the conclusion that dilution of the sample with water before the assay, combined with vortexing, is an efficient way of disaggregation (Dawney AB. et al. 1982).

In summary, these data indicate that reliable uromodulin measurements can be obtained from untreated urine samples, provided they are immediately stored at -80 °C and assayed within 3 months, with vortexing and dilution with water to prevent aggregation. This methodology will be useful for high-throughput analyses of uromodulin and its validation as a biomarker for renal function and risk of CKD.

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IV. Common UMOD Variants Influence Urinary and Plasma Levels and Biochemical Properties of Uromodulin

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Abstract

Uromodulin is exclusively expressed in the kidney and is the most abundant protein in normal urine. Common variants located in the promoter of the *UMOD* gene encoding uromodulin are associated with the excretion of uromodulin, and with the risk of chronic kidney disease and hypertension. The potential impact of the *UMOD* variants on plasma level and on biochemical properties of uromodulin remains unknown.

The potential influence of the *UMOD* promoter variant rs12917707 on the urine and plasma levels of uromodulin (ELISA) was analyzed in two Swiss urban cohorts (n = 6'076). Qualitative analyses were carried out by immunoblotting, 2D-gel electrophoresis and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) on urine samples from age- and sex-matched homozygous carriers of protective or risk *UMOD* variants

The common, risk allele of rs12917707 was associated with higher urinary uromodulin levels irrespective of normalization to creatinine, and with higher plasma levels of uromodulin independent of age, gender and eGFR. The plasma levels of uromodulin were ~1000-fold lower than urine levels. The rs12917707 variant influenced the immunoreactivity of uromodulin, depending on N-glycosylation pattern. These differences were confirmed by 2D-gel electrophoresis and substantiated by distinct glycan compositions as observed in MALDI-MS. These data show that common variants in the *UMOD* promoter region exert a strong influence on the urine and plasma levels of uromodulin and are associated with significant biochemical modifications of the protein. These results provide insights into the regulation of uromodulin and its biological properties.

Introduction

Uromodulin (Tamm-Horsfall protein) is the most abundant urinary protein under normal physiological conditions. This complex protein is exclusively produced by tubular cells in the thick ascending limb (TAL) of the loop of Henle, and is secreted into the urine at a rate of 50 - 100 mg per day. Uromodulin, which belongs to the family of zona pellucida (ZP) proteins, contains 48 cysteine residues involved in the formation of 24 disulfide bonds (Rampoldi L. et al. 2011) as well as 7 potential N-glycosylation sites and a C-terminal glycosylphosphatidylinositol anchor attachment. O-linked glycosylation of uromodulin has also been reported (Easton RL. et al. 2000), Uromodulin is encoded by the *UMOD* gene located on chromosome 16p12.3, under the control of a promoter whose proximal part is highly conserved in mammalian species (Zhu X. et al. 2002).

The biological role of uromodulin remains elusive. The protein is cleaved and released from the apical membrane of the TAL cells into the urine, where it is assembled into large, polymeric fragments. Uromodulin interacts with many urine components *in vitro*, and investigations based on the *Umod* knock-out (KO) mouse models revealed that it may protect against urinary tract infections through binding to type I fimbriated *Escherichia coli* (Bates JM. et al. 2004) and inhibit the formation of kidney stones by reducing the aggregation of calcium oxalate crystals (Liu Y. et al. 2010). Recently, mutations in *UMOD* have been associated with autosomal dominant tubulointerstitial kidney disease (ADTKD-UMOD) (Eckardt KU. et al. 2015), a rare condition characterized by hyperuricemia, alteration of urinary concentrating ability, and tubulointerstitial fibrosis leading invariably to chronic renal failure. Furthermore, genome wide association studies (GWAS) consistently reported a significant association between common variants located in the promoter region of *UMOD* (e.g. rs12917707 G > T or rs4293393 T > C) and renal function parameters, risk of CKD, kidney stones and hypertension. Initial studies by Köttgen et al. suggested that the *UMOD* promoter variants may modulate uromodulin levels, with risk allele carriers showing significantly higher uromodulin levels in urine (Köttgen A. et al. 2010). The direct role of the *UMOD* promoter variants was demonstrated by Trudu et al. who showed that risk alleles were significantly associated with increased expression and transcription of *UMOD* gene, both *in vivo* and *in vitro* (Trudu M. et al. 2013). Of interest, these variants were shown to activate the NKCC2 cotransporter in the TAL, causing a salt-sensitive increase in blood pressure, and to cause progressive lesions in the kidney (Trudu M. et al. 2013).

In a meta-GWAS encompassing 10,884 European subjects, Olden et al. showed a major effect of the *UMOD* promoter variants on the urinary levels of uromodulin (Olden M. et al. 2014). If the regulatory effect of *UMOD* promoter variants on the transcription and urinary levels of uromodulin is well established, the potential influence of these variants on the biochemical features of uromodulin and on its circulating levels are unknown. These questions are important, when considering that the lead *UMOD* promoter variants regulating uromodulin expression are located in a large LD block comprising exons 3, 4, and 6, containing the N-glycosylation sites. Possibly, genotype-related changes in glycosylation patterns may affect the interactions of uromodulin with uropathogenic *Escherichia coli* (*E. coli*.) Despite recent evidence linking urine uromodulin levels with systemic inflammation, potential correlations between urine and circulatory levels of uromodulin have not been investigated (Ghirotto S. et al. 2016).

In this study, we investigated the potential impact of a common promoter variant of *UMOD* on urine and plasma level and on biochemical properties of uromodulin in two Swiss urban cohorts. Qualitative analyses were carried out to substantiate biochemical modifications of uromodulin obtained from homozygous carriers of protective or risk *UMOD* variants.

Material and Methods

Urine and plasma samples*:* The effect of *UMOD* genotype on urine uromodulin levels were analyzed in spot urine samples from the Swiss cohort CoLaus (n = 5'126). The CoLaus Study is a population-based cross-sectional study of more than 5000 participants (52.5 % women) aged 35-75 years living in Lausanne, Switzerland. Clinical and biological data were collected on each participant including spot urine after an overnight fast (Firmann M. et al. 2008). The uromodulin immunoreactivity and the pattern of N-glycans with regards to *UMOD* genotype were analyzed in a subset of CoLaus samples selected for the rs12917707 genotype and matched for age, gender and creatinine-based estimated glomerular filtration rate (eGFR) eGFR was calculated with the CKD-Epidemiology Collaboration (CKD-EPI). The comparison between plasma and urine uromodulin levels was performed on samples from the Swiss kidney Project on Genes and Hypertension (SKIPOGH). In this family-based multi-centre cross-sectional study clinical and biological information were collected on participants including 24 h urine (day-time urine and night-time urine were collected in separate containers) and plasma (Ponte B. et al. 2014). We used day-time urine and plasma samples, obtained the same day, and matched for age, gender

and eGFR.MALD-MS experiments were carried out on freshly collected second morning urine samples from 2 men age-matched with divergent genotype.

Urine collection protocols for uromodulin MALD-MS experiments have been approved by the ethical committee of the Université Catholique de Louvain. For the two Swiss population-based studies CoLaus and SKIPOGH protocols were approved by local ethical committees. All participants provided written informed consent (Pruijm M. et al. 2016).

Uromodulin measurements: Urine and plasma uromodulin were measured by a validated in-house ELISA as previously described (Youhanna S. et al. 2014; Olden M. et al. 2014). In brief, we used a sheep anti-human uromodulin antibody (K90071C; Meridian Life Science, Memphis, TN) as the capture antibody, a mouse monoclonal anti-human Tamm-Horsfall protein antibody (CL 1032A; Cedarlane Laboratories, Burlington, NC) as the primary antibody, and a goat anti-mouse IgG (H+L) horseradish peroxidase–conjugated protein (172.1011; Bio-Rad Laboratories, Inc., Hercules, CA) as a secondary antibody. Standard curve was established using human uromodulin (AG 733, stock solution: 100 µg/ml; EMD Millipore, Temecula, CA). This assay has a sensitivity of 2.8 ng/ml and a linearity of 1.0. The inter-assay and intra-assay variability are 3.28 % and 5.46 %, respectively and the detection range is between 3.9 and 500 ng/ml. We diluted urine samples 1:101 in double distilled water whereas plasma samples were used without any dilution. A commercially available ELISA kit (Euroimmun AG, Lübeck, Germany) was also used to measure plasma uromodulin levels following the manufacturer's instruction.

Biochemical treatments: De-N-glycosylation was performed on human urine using a set of enzymes according to manufacturer's instructions. The following enzymes were used: Peptide-N-Glycosidase F (PNGaseF) (New England Biolabs, P0704L) for removal of high mannose, hybrid, and complex N-glycans, Neuraminidase (sialidase) (New England Biolabs, P0720S) to catalyze the hydrolysis of N-acetyl-neuraminic acid residues and Endoglycosidase H (New England Biolabs, P0702S) to cleave within the chitobiose core of high mannose and some hybrid type N-glycans from glycoproteins.

Immunoblotting: Sodium dodecyl sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) was performed under reducing conditions on human urine samples. Samples were loaded after being mixed with Laemmli sample buffer (Bio Rad, USA) and heated for 5 min at 95 °C. Proteins were separated on 10 % SDS-PAGE and transferred to polyvinyl difluoride (PVDF, Bio Rad Laboratories, USA) membrane (activated with 90 % methanol for 2 min) for Western

blotting. Membranes were blocked with 5 % milk for 30 min at room temperature then incubated overnight at 4 °C with either sheep anti-human uromodulin antibody (Meridian Life Science, Memphis, TN, USA; K90071C) or mouse monoclonal anti-human uromodulin antibody (Cedarlane Laboratories, Burlington, NC, USA; CL 1032A) (1:500 in 0.5 % BSA blocking buffer). Secondary antibodies were applied for 1 hour at room temperature. We used goat anti-mouse horseradish peroxidase (HRP) conjugated (1:10,000) (P0447 Dako, Denmark), or polyclonal rabbit anti-sheep HRP conjugated (1:1000) (P0163, Dako, Denmak). Antigen-antibody reaction was detected by using enhanced chemiluminescence (Immun-Star HRP, Bio-Rad) and light-sensitive film (GE Healthcare, Glattbrugg, Switzerland). The molecular weight was estimated by running the Precision Plus Protein™ All Blue standard (Bio-Rad).
Two-dimensional gel electrophoresis: Urine samples were diluted in 8 M urea, 2 M thiourea, 4 % CHAPS, 0.05 % Zwittergent, 3 mM TCEP, 100 mM DeStreak and 0.5 % IPG buffer pH 3-5.6. The samples were loaded on Immobiline Dry strip pH 3-5.6, 7 cm for the first dimension (total focusing run was 50,000 Vh). IPG strips were sequentially reduced and alkylated prior to the 2nd dimension electrophoresis with SDS-PAGE 7.5 %. Proteins were electroblotted to nitrocellulose membrane for 2 h at 390 mA. Membranes were blocked overnight in TBS-T buffer containing 5 % milk.

Uromodulin N-glycans isolation and analysis: To retrieve and study uromodulin N-glycans we adapted the following procedure: first we precipitated uromodulin from urine following the protocol of Tamm and Horsfall (Tamm I. et al 1950), and then we isolated uromodulin on coomassie-stained 10 % SDS-PAGE. Uromodulin bands were incubated with PNGase F enzyme (100 U/ml in 20 mM ammonium bicarbonate) overnight at 37 °C as described by Küster et al. (Küster B. et al. 1997) the second day supernatant containing N-glycans was collected and stored in -20 °C until MALDI-MS analysis.

Matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS) Uromodulin N-glycans isolated from both genotypes were permethylated using the sodium hydroxide procedure, as described previously and MALDI-TOF-MS data were acquired using an ABSciex 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems) (Hülsmeier AJ. et al. 2011).

Data analysis: Quantification of immunoblotting bands was done using ImageJ1 software. Nycthemeral data curve fitting was performed on Acro 3.5. Statistical Package for

Social Sciences (SPSS) version 19 (IBM Corp., Armonk, NY, USA) was used for curves distribution, Pearson correlation coefficient, paired t-test and analysis of variance (ANOVA). Continuous data reported as mean ± SEM.

The N-glycans were annotated from the MALDI MS spectra using the Cartoonist algorithm (Goldberg D. et al. 2005). Peak lists were exported from the Data Explorer Software Version 4.9 (Applied Biosystems) to MS-Excel. The areas of the identified N-glycan signals were normalized to the sum of all glycan signals, and the standard deviations for the relative peak areas were computed using Graph Pad Prism Version 6.01. Six (GG genotype) and seven (TT genotype) technical replicates were analyzed.

Results

UMOD promoter variant and urinary levels of uromodulin in the general population

The *UMOD* promoter variants (rs12917707 and rs4293393) have consistently been associated with the risk for CKD and hypertension in the general population. These variants are located in a large linkage disequilibrium (LD) block that encompasses exons 3, 4 and 6 where N-glycosylation sites are encoded (Figure 1).

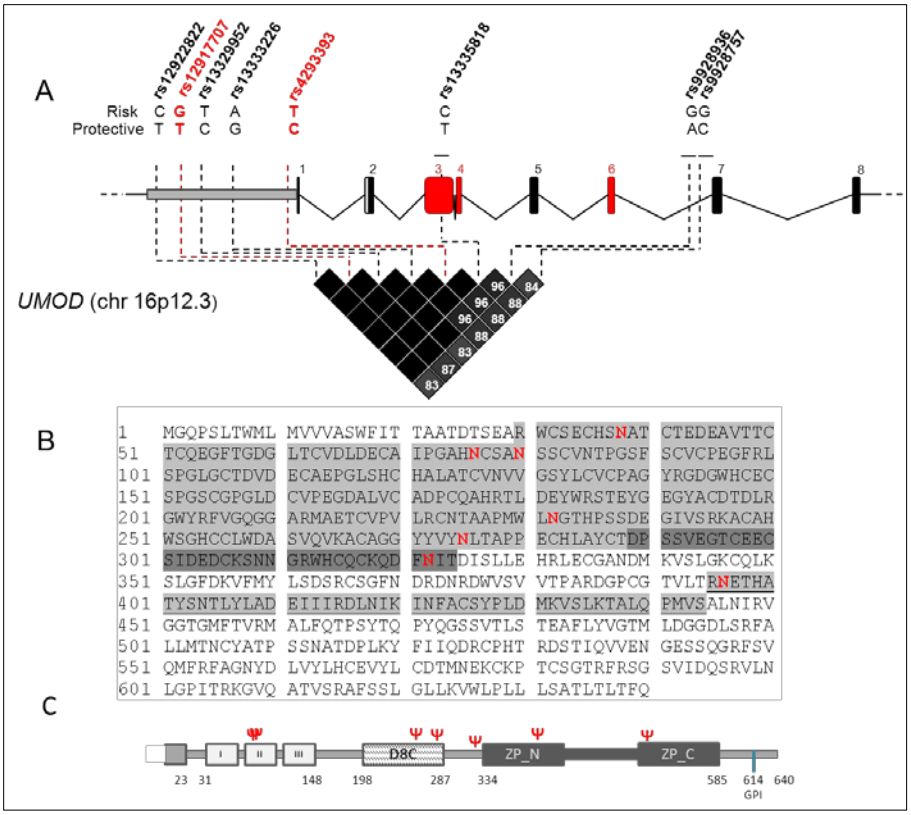

Figure 1: Variants in the UMOD locus and structure of uromodulin.a) UMOD promoter variants 12917707 and 4293393 are a LD block encompassing other variants from exons 3, 4 and 6. b) Amino acid sequence of human uromodulin with N-glycosylation sites in red. Exons are indicated as follow: exon 3 highlighted in grey, exon 4 highlighted in dark grey and exon 6 highlighted in grey and underlined. c) The structure of uromodulin consists of 3 epidermal growth factor (EGF)-like domains, a central domain named D8C it contains eight conserved cysteines, a zona pellucida (ZP) domain, and a glycosylphosphatidylinositol (GPI)-anchoring site. N-glycosylation sites are indicated in red.

We analyzed the effect of these variants on uromodulin levels in spot urine samples from the CoLaus population (n = 5,126) (Figure 2).

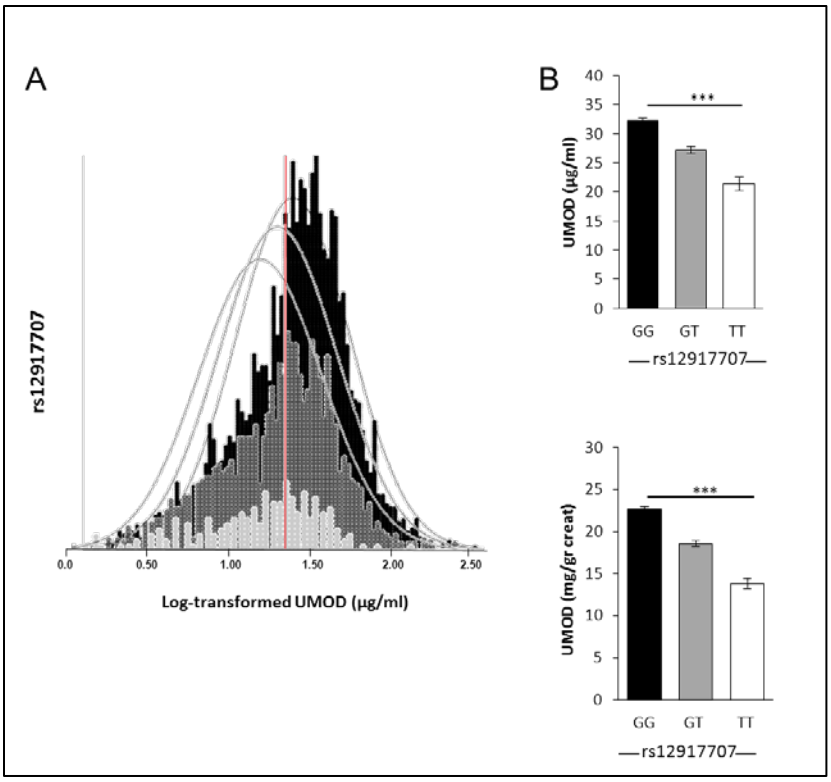

Figure 2: Effect of UMOD variant on uromodulin levels. a) Normal distribution of log-transformed urine uromodulin levels in a Swiss cohort CoLaus among genotype categories of UMOD variant rs12917707 GG n = 3,463, GT n = 1,502, TT n = 197. The red line indicated the mean of uromodulin in the general population (31.03 ± 0.38 µg/ml; n = 5,126). b) Average urine uromodulin levels (absolute and normalized to creatinine) in genotype categories. GG: 33.23 ± 0.51 µg/ml; 22.73 ± 0.26 mg/g creatinine, GT: 27.23 ± 0.58 µg/ml; 18.60 ± 033 mg/g creatinine, TT: 21.36 ± 1.17 µg/ml; 13.86 ± 0.63 mg/g creatinine. Uromodulin levels (absolute and normalized to creatinine) were significantly different among genotype categories ($p < 0.001$). *** $p < 0.001$.

The distribution of uromodulin based on the rs12917707 *UMOD* genotype is normal in all categories (Figure 2A). The average values of uromodulin (absolute and normalized to creatinine, respectively) are: general population (31.03 ± 0.38 µg/ml; 21.19 ± 0.22 mg/g creatinine; N 5,126); GG (risk) carriers (33.23 ± 0.51 µg/ml; 22.73 ± 0.26 mg/g creatinine; N = 3,463); GT carriers (27.23 ± 0.58 µg/ml; 18.60 ± 033 mg/g creatinine; N = 1’502); and TT (protective) carriers (21.36 ± 1.17µg/ml; 13.86 ± 0.63 mg/g creatinine; N = 197) (Figure 2B). These data demonstrate the major effect of the rs12917707 variant, with a dose dependent effect of the G allele on the levels of uromodulin in urine. As compared to the homozygous TT (protective) carriers, the homozygous GG (risk) carriers have a 2-fold increase in the indexed

uromodulin levels in urine, whereas the heterozygous GT carries show intermediate levels. Changes of uromodulin levels among genotype categories were significant ($p < 0.001$).

Influence of UMOD promoter variant on plasma uromodulin levels

We investigated the effect of the *UMOD* variant rs12917707 on the level of circulating and urinary uromodulin in samples from the urban Swiss SKIPOGH cohort matched for age, gender and eGFR (Table 1).

	Low uUMOD (N = 23)	**High uUMOD (N = 23)**	**P values**
Age	53.5 ± 3.23	49.4 ± 3.05	0.54
uUMOD (µg/ml)	6.62 ± 0.90	80.35 ± 7.81	< 0.01
uUMOD (mg/g creat)	10.7 ± 1.59	65.8 ± 10.7	< 0.01
pUMOD (ng/ml)	23.1 ± 2.36	34.1 ± 3.26	0.013
eGFR (mL/min/1.73 m^2)	90.2 ± 3.73	98.0 ± 3.38	0.22

Table 1 A: Plasma and urine levels of uromodulin levels in 23 pairs of samples matched for age, gender and eGFR form the general population (SKIPOGH). Gender distribution: 11M/12F. u: urine; p: plasma.

	TT (N = 21)	**GG (N = 21)**	**P value**
Age	53.4 ± 3.22	54.3 ± 3.27	
uUMOD (µg/ml)	14.2 ± 2.87	31.7 ± 4.33	< 0.01
uUMOD (mg/g creat)	17.2 ± 2.76	40.5 ± 4.65	< 0.01
pUMOD1 (ng/ml)	10.6 ± 1.73	17.6 ± 1.24	< 0.01
pUMOD2 (ng/ml)	34.5 ± 3.22	59.4 ± 4.16	< 0.01
eGFR (mL/min/1.73 m^2)	91.3 ± 3.60	91.6 ± 3.71	0.95

Table 1 B: Plasma and urine uromodulin levels in GG vs TT (rs12917707) categories in SKIPOGH samples. pUMOD1: plasma uromodulin levels measured with In-house assay (Youhanna S. et al. 2014); pUMOD2: plasma uromodulin levels measured with commercial assay (Euroimmun AG, Lübeck, Germany).

Gender distribution: 8M/13F Subjects homozygous for the G (risk) allele showed significantly higher levels of uromodulin in the urine (both absolute and indexed to creatinine) and in the

plasma. These differences were observed when using either our well-characterized ELISA or with a commercial kit, and with normal eGFR values.

The potential link between the urine and plasma levels of uromodulin was further analyzed in a subset of subjects from SKIPOGH. A large variation in urine uromodulin, independent of the genotype, was observed in this cohort, the 5^{th} and 95^{th} percentile values being 5.58 µg/ml (5.87 mg/g creatinine) and 58.3 µg/ml (69.46 mg/g creatinine) respectively. We measured plasma uromodulin level in two subgroups matched for age and eGFR but with divergent urine uromodulin levels (low urine level: 6.67 ± 0.86 µg/ml; 10.6 ± 1.53 mg/g creatinine, n = 23; and high urine level: 80.3 ± 7.81 µg/ml (65.8 ± 10.75 mg/g creatinine, n = 23) (Table 1). Individuals in the high urine uromodulin category showed significantly higher plasma uromodulin levels (34.05 ± 3.26 ng/ml compared to 23.73 ± 2.31ng/ml; P = 0.013).

Effect of *UMOD* variant on uromodulin immunoreactivity and N-glycosylation

The rs12917707 variant is located in an LD block comprising exons 3, 4 and 6 that code for Asn residues involved in N-glycosylation sites, suggesting that this variant may affect either the glycosylation site usage or the maturation of N-glycans along the secretory pathway.

We investigated the potential effect of the rs12917707 *UMOD* variant on the biochemical properties of uromodulin in urine samples from CoLaus (10 pairs of human urine samples GG vs TT) matched for age, gender and eGFR, using 3 different anti-uromodulin antibodies. Immunoblotting experiments were done by loading the same amount of uromodulin (based on ELISA measurements) for all samples (Figure 3).

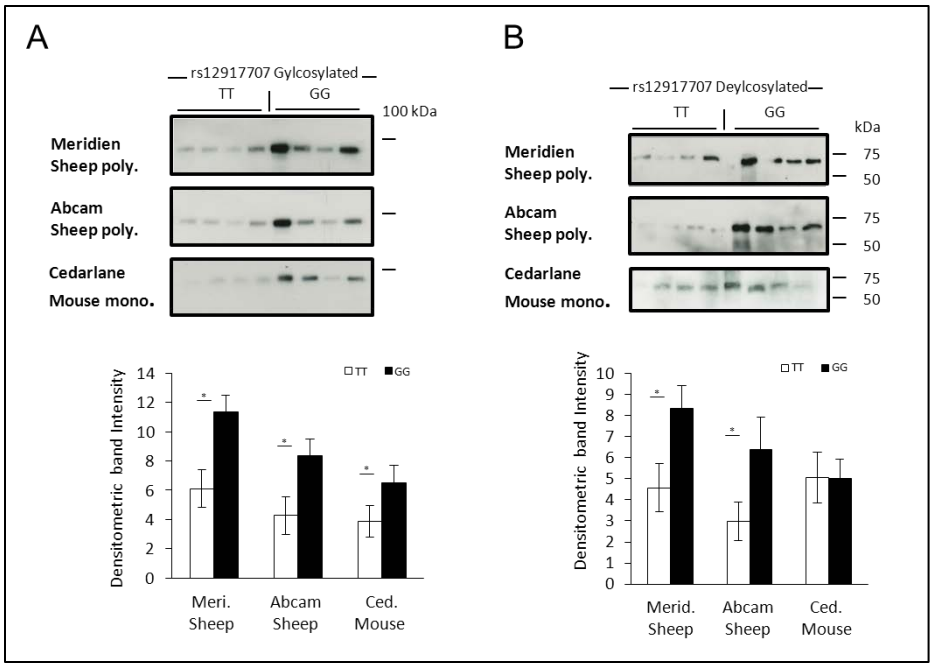

Figure 3: Uromodulin immunoreactivity among UMOD genotypes (rs12917707). Representative western blot analysis (10 % SDS-PAGE) of human urine samples (10 pairs) (5ng/well) showing uromodulin immunoreactivity among genotype categories of UMOD rs12917707 with different anti-uromodulin antibodies (Meridien and Abcam are polyclonal sheep anti-uromodulin (1:500) and Cederlane is monoclonal mouse anti-uromodulin (1:500)): a) Mature (N-glycosylated) uromodulin immunoreactivity. Significant difference observed between genotypes in antibodies categories. b) Peptide-N-Glycosidase F deglycosylated uromodulin immunoreactivity among UMOD genotypes (rs12917707). Significant difference observed between genotypes in antibodies categories expect for Cedarlane mouse antibody. * $p < 0.05$.

Samples harbouring GG genotype of rs12917707 consistently showed higher signal intensity than the TT samples with the 3 antibodies when quantifying glycosylated uromodulin bands (Figure 3A). Of interest, these systematic differences in immunoreactivity were no longer observed after treatment with PNGase F to remove the totality of N-glycans. Assessment of bands intensity of deglycosylated protein shows uniform signal using the mouse monoclonal antibody while the difference among genotype remains unchanged with the two polyclonal antibodies (Figure 3B). These data suggest that difference of immunoreactivity can be explained at least in part by a difference in the N-glycans reflecting the two different *UMOD* genotypes. To gain insight into the glycan modifications responsible for the different immunoreactivity, selected urine samples were subjected to two additional treatments affecting the N-glycan composition. Sialidase was used to remove sialic acid and Endo H to remove glycans that have

not been modified in the Golgi (high mannose) reported only on 1 site on mature uromodulin. We observed a difference in immunoreactivity between genotypes (GG vs TT) as well as within the same genotype category.

The observed modification in immunoreactivity among genotypes has been additionally investigated using two-dimensional gel electrophoresis (2-DGE) on two pairs of human urine samples selected from the above-mentioned samples. Analysis showed different isoelectric point (IP) among genotypes in the fully glycosylated state (Figure 4).

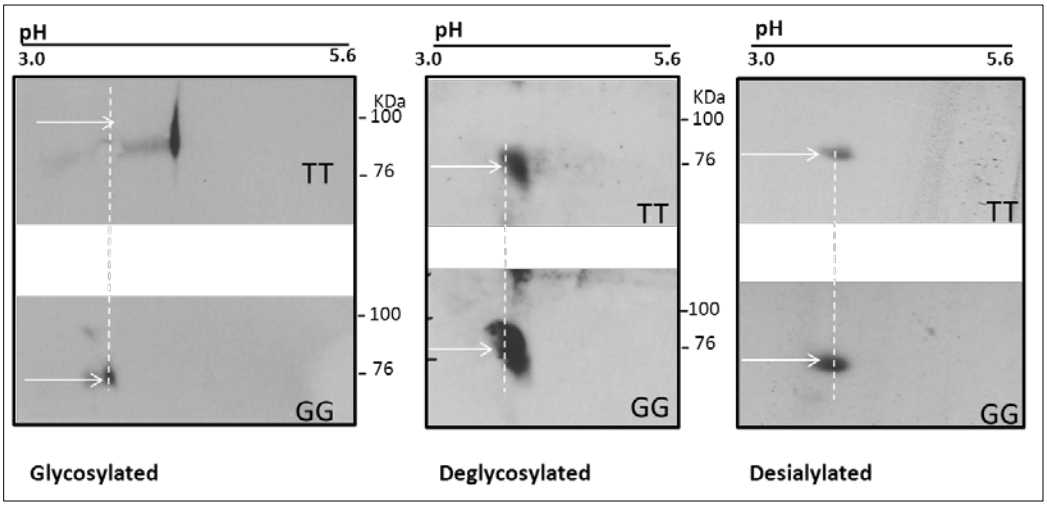

Figure 4: A representative two-dimensional gel (2D gel) analysis for uromodulin among UMOD genotypes (rs12917707). Comparison of the isoelectric point of mature uromodulin (fully glycosylated) versus de-N-glycosylated and desialylated uromodulin was performed in 2 pairs of human urine samples. Human urine samples were treated with PNGase F or sialydase (following manufacturer's protocol) and loaded on a 2D gel.

Samples with the GG genotype displayed lower IP than those with the TT genotype. This difference was abolished after removal of N-glycosylation with PNGase F or desialylation with sialidase, indicating that increased sialylation on N-glycans contributes to the acidic IP of uromodulin of the GG genotype.

MALDI-MS analysis of uromodulin glycans

N-glycans were released from uromodulin by in-gel digestion with PNGase F and analyzed by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS). The N-glycan profiles of the GG- and TT-genotypes were compared and the identified glycans were in agreement with glycosylation profiles described previously (Halim A. et al. 2012; Hong CY. et

al. 2013). A tendency of increased relative amounts of tetra-antennary, tetra-sialylated N-glycans in the GG-genotype related uromodulin was apparent (Figure 5A).

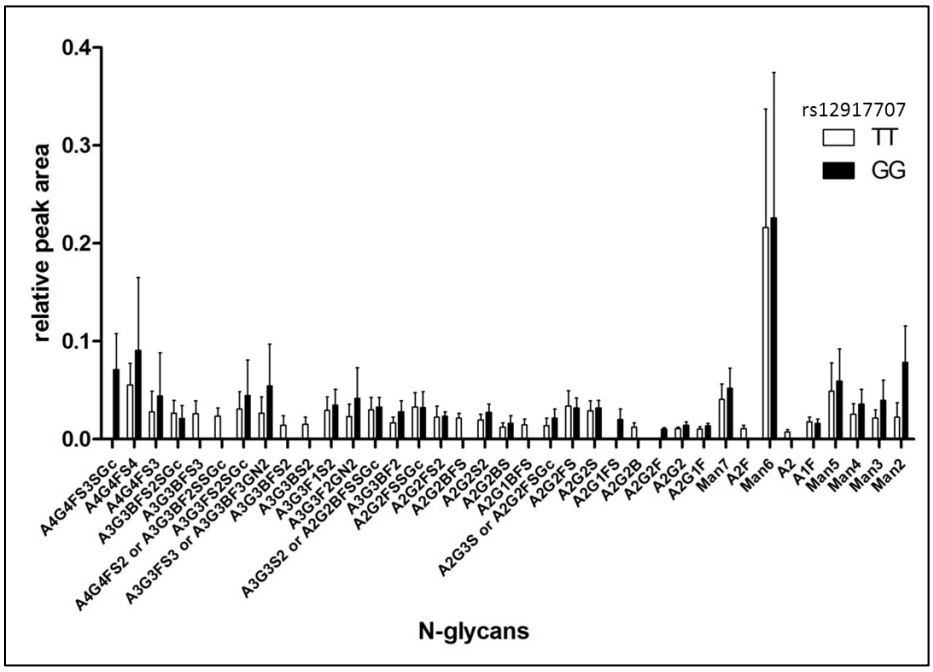

Figure 5A: Uromodulin N-glycans profile among UMOD genotypes (rs12917707). A comparison profile of uromodulin N-glycans of GG vs TT samples. The average relative peak areas for each glycan are plotted (error bars indicate SEM). Glycans detectable at least 3 times were normalized to the sum of peak areas within a MALD-MS acquisition.

Nevertheless, a statistically significant difference between the genotypes could not be calculated, due to variability in glycan compositions between samples analyzed. We observed an overall difference in the uromodulin N-glycan compositions between the genotypes, with some glycans being exclusively present in the TT- or GG-genotype (Figure 5B).

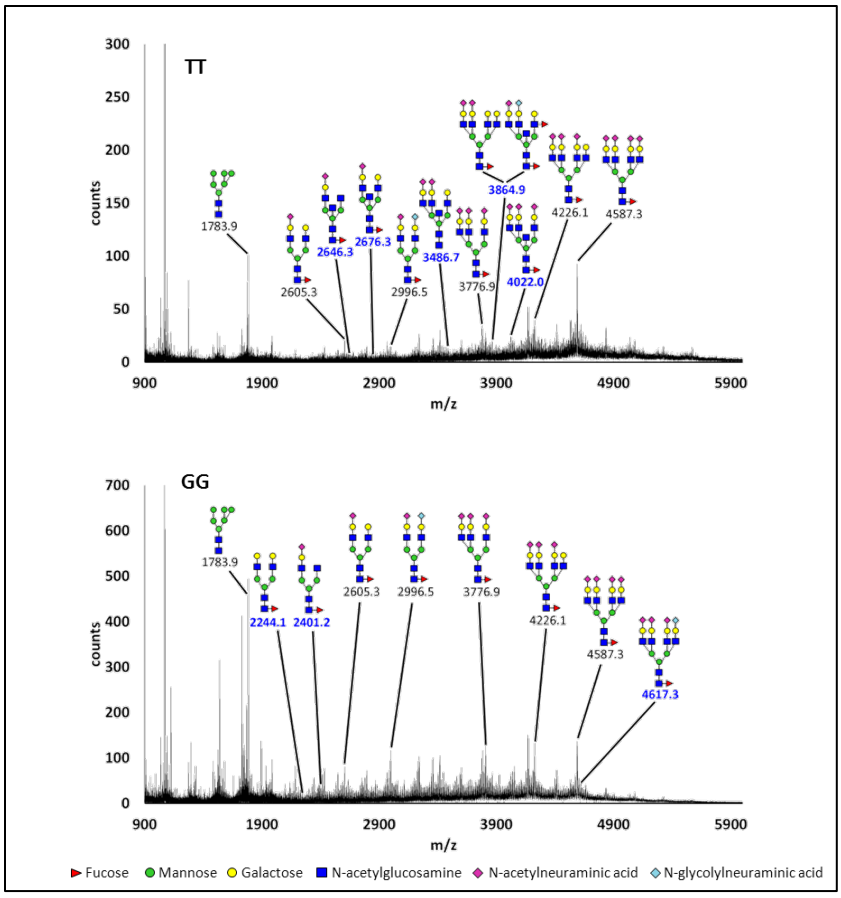

Figure 5B: Uromodulin N-glycans profile among UMOD genotypes (rs12917707). A representative MALDI-MS profile of permethylated N-glycans from urinary uromodulin GG and TT subjects (1 pair). Glycans highlighted in dark blue are only detected in either TT sample or in GG sample.

Discussion

In this study, we show for the first time that a common variant in the promoter of *UMOD* (rs1297707), consistently associated with the risk of CKD and hypertension in the general population, mediates the urinary excretion of uromodulin and also affects the levels of circulating uromodulin. The latter is approximately 1000-fold lower than the urine levels, which were shown to follow a nycthemeral cycle. The *UMOD* variant, located in a large LD block covering 6 exons of the gene, is associated with changes in the immunoreactivity of uromodulin, secondary to modified N-glycosylation composition. These biochemical changes were not caused by modifications in the coding sequence of *UMOD*.

The common variant of *UMOD* promoter rs12917707 is in total LD with other *UMOD* promoter variants of which major alleles have been identified as risk alleles for several renal diseases such

as chronic kidney disease (CKD), kidney stones and hypertension in different genome wide association studies (GWAS) (Köttgen A. et al. 2009; Köttgen A. et al. 2012; Padmanabhan S. et al. 2010; Reznichenko A. et al. 2012). As risk alleles have been linked to elevated urinary uromodulin levels in the general population (Olden M. et al. 2014), higher uromodulin levels were consequently linked to higher predisposition to previously mentioned renal disorders. In this study we confirm that the distribution of urinary uromodulin levels, whether normalized to creatinine or not, is genotype-dependent: risk allele G accounts for higher uromodulin levels than minor allele T.

Our efforts to investigate modulators of plasma uromodulin levels in an age-matched group with normal renal function proved that *UMOD* variant rs12917707 affects significantly variations in plasma uromodulin. Higher plasma uromodulin levels are observed within subjects displaying the major allele G and high urinary uromodulin. Very few inconsistent studies have investigated serum uromodulin levels as a marker for renal function (Risch L. et al. 2014) or for disease progression (Prajczr S. et al. 2010) but so far any study had interrogated plasma uromodulin changes with regards to an important modifier of urinary uromodulin *UMOD* variant.

We think that elevated urinary and plasma uromodulin observed in the presence of risk allele may result from early damage in the tubular interstitium thus preceding TAL damage. These novel findings correlating urinary uromodulin levels to plasma uromodulin together with genotype influence of *UMOD* promoter variant on both uromodulin levels may give an insight on the role of this variant.

The SNPs within the promoter region of uromodulin gene are an LD block encompassing exons 3, 4 and 6. These exons encode for N-glycosylation sites which are essential for functions attributed to uromodulin, mainly the defense against urinary tract infections through binding to type I fimbriated *Escherichia coli* via its high-mannose residues (Bates JM. et al. 2004). In an attempt to elucidate the potential mechanisms of the relationship between *UMOD* promoter variants and renal dysfunction we scrutinized uromodulin glycosylation patterns among SNP genotypes using samples with divergent genotype and employing several technics. We observed genotype-dependent variations in signal detection and IP; samples exhibiting the minor allele T display lower immunoreactivity and higher IP compared to samples with the major allele G. These discrepancies between genotypes were abolished after de-N-glycosylation indicating that

differences in glycan composition may explain the perceived divergence. Additionally, N-glycan profiling by MS-MALDI in a pair of human urine samples matched for age and gender but genotype divergent indicates that indeed N-glycan composition differs among genotypes. Of note, this difference is not an outcome of variation in food composition rather an outcome of cellular synthesis. Recognizing the importance of N-glycans in the protective role of uromodulin against urinary tract infection, toxic metabolite in the urine (Parsons CL. et al. 2011) and kidney stone formation (Viswanathan P. et al. 2011) thus alteration of glycan on uromodulin surface may have considerable consequences on protein functionality. Considering these data, together with the effect of *UMOD* variant on urinary uromodulin levels, we hypothesize that modification of glycan composition could be a process by which cellular machinery adapts to the increased production of this glycoprotein in the case of risk allele. Further investigations are needed to better explore the effect of *UMOD* variant on uromodulin N-glycosylation and its consequences on its function particularly that the risk allele is present in high frequency in the general population.

We acknowledge that this study has limitations. We have investigated glycans composition among genotypes by MALDI-MS only in one pair of subject. This is due to the difficulty in finding subjects homozygous for minor allele of UMOD promoter variant since it has a genotype frequency of 4 % in the general population.

The advantages of this work are: homogeneous sample size, correlations plasma and urine, morning samples, optimalized for uromodulin ELISA, genotype known, matching for age, gender and eGFR, large cohorts allowing comparison of the rare homozygous state for protective variants.

In conclusion, our study provides novel evidence that *UMOD* promoter variant rs12917707 influences the excretion of plasma uromodulin and the biochemical property of urine uromodulin, thus giving insights into mechanisms how this variant leads to renal disorders.

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V. Uromodulin excretion: Association with clinical and genetic factors in the general population

This chapter is a summary of the following published articles:

1-Associations of Urinary Uromodulin with Clinical Characteristics and Markers of Tubular Function in the General Population.

Pruijm M., Ponte B., Ackermann D., Paccaud F., Guessous I., Ehret G., Pechère-Bertschi A., Vogt B., Mohaupt MG., Martin PY., Youhanna SC., Nägele N., Vollenweider P., Waeber G., Burnier M., Devuyst O., Bochud M. *Clin J Am Soc Nephrol*. 2016; 7; 11 (1): 70-80.

2-Clinical, Genetic, and Urinary Factors Associated with Uromodulin Excretion.

Troyanov S., Delmas-Frenette C., Bollée G., Youhanna S., Bruat V., Awadalla P., Devuyst O., Madore F. *Clin J Am Soc Nephrol*. 2016; 7; 11 (1): 62-9.

Introduction

Uromodulin is the most abundant protein in the urine under normal physiological conditions and it is secreted exclusively by the epithelial cells of the thick ascending limb (TAL). Several biological roles have been attributed to this protein, recent studies showed an association between uromodulin secretion and the regulation of sodium transport in the thick ascending limb (TAL) (Trudu M. et al. 2013; Mutig K. et al. 2011), other preliminary studies have suggested an association between uromodulin and estimated glomerular filtration (eGFR) but these findings still lack the confirmation within cohorts of large sample size (Thornely C. et al. 1985; Reinhart HH. et al. 1991).

Mutations in the *UMOD* gene have been shown to cause uromodulin-associated kidney diseases (UAKD). UAKD are autosomal dominant disorders characterized by hyperuricemia and gout early in life, alteration of urinary concentrating ability, and tubulointerstitial fibrosis with occasional cysts at the corticomedullary junction. UAKD invariably lead to chronic renal failure during the third to seventh decade of life. The identified mutations (mostly missense, affecting cysteine residues) affect the biosynthesis of uromodulin and lead to its accumulation in the endoplasmic reticulum (ER) thus decreased uromodulin excretion. These events may cause ER stress and tubulointerstitial injury leading eventually to progressive renal damage (Rampoldi L. et al. 2011). In addition to its direct involvement in UAKD, recent genome wide association studies (GWAS) pointed at uromodulin as a risk factor for CKD, kidney stones, and hypertension (Köttgen A. et al. 2012). Nevertheless, the identification of common and rare variants of *UMOD*, associated with common traits and monogenic diseases of the kidney, points toward the high relevance of uromodulin biology.

In this work, we use clinical, biological and genetic information from several large cohorts to better understand the function and the clinical significance of uromodulin including factors influencing the glycoprotein excretion and its relationship with eGFR.

Material and Methods

Studied populations and functional parameters

Our work analyses and study investigations were conducted in 3 populations. Two Swiss population-based studies: the Swiss Kidney Project on Genes and Hypertension (SKIPOGH) (2009-2012). n = 817; the Cohort Lausannoise (CoLaus) (2003-2006) n = 5,706 and a subset of individuals chosen from a Canadian cross-sectional study CARTaGENE (CaG) (2009-2015) n = 46. Individuals in the SKIPOGH study were randomly selected from the general population in Bern, Lausanne and Geneva. Participants were adult and European descent. Clinical and demographic data were collected from all participants in addition to complete physical examination, blood sampling after overnight fasting, 24 h urine collection and renal ultrasonography (Ponte B. et al. 2014). Participants in the CoLaus study were adults from Lausanne having a European descent. Anthropometric data were collected from participants in addition to morning spot urine and blood sampling after overnight fasting (Firmann M. et al. 2008). The CARTaGENE study included participants from Quebec 40-69 years old. At the study visit medical history (including medication usage information) was gathered from all participants in addition to physical examination, blood and morning spot urine collection (Awadalla P. et al. 2013). All study protocols were approved by local ethical committees. The baseline characteristics for the study participants are shown in Table 1.

We analyzed clinical and biological correlates of uromodulin excretion as well as its association with markers of glomerular and tubular function in SKIPOGH and CoLaus. We also established and confirmed associations of uromodulin levels with relevant parameters in serum and urine in addition to patients' medical history within CARTaGENE individuals.

Renal ultrasound was performed on SKIPOGH participants according to a standardized procedure described by Pruijm M. et al. (Pruijm M. et al. 2013) Renal volume was calculated as 0.523 x length x width x transverse diameter (Jones TB. et al. 1983). All ultrasounds were performed by one experienced operator at each center.

Laboratory measurements were carried out using standard clinical laboratory methods. Serum measurements included: urea, creatinine, electrolytes, uric acid, and glucose. Urinary measurements included: glucose, electrolytes, uric acid, creatinine, osmolality and albumin. The CKD-EPI formula was used to calculate eGFR (Levey AS. et al. 2009).

In SKIPOGH, a 24 h urine collection was started on the day of the study visit and returned the next day by the participant. Incomplete urine collection was defined as a volume less than 300 ml per 24 h, a 24 h urinary creatinine excretion of less than 0.1 mmol/kg body weight, or if reported as incomplete by the participant. Urine collections containing > 0.4 mmol/kg creatinine were also excluded from further analysis (Bankir L. et al. 2008).

In CoLaus and CARTaGENE, urinary uromodulin, creatinine, electrolytes concentrations and osmolality were measured in morning spot urine samples.

Urinary biochemical parameters were measured from samples stored at -80 °C, using the same biochemical platform at the University of Zurich. The 24 h urinary osmolar excretion in mosm was calculated as: 24 h osmolality x 24 h urinary volume.

Uromodulin was measured from urinary samples stored at -80 °C at the University of Zurich (Youhanna S. et al. 2014). Urinary creatinine levels were measured using Beckman Coulter Synchron System Creatinine Assay (Unicell DxC Synchron Clinical System) following the manufacturer's instructions.

Individuals from the CARTaGENE population with genotype data were selected from an ongoing sub-study on common variant associations in cardiovascular disease: top 150 and bottom 150 Framingham scores for both men and women (n = 600) and top and bottom 100 patients on the basis of the vascular rigidity index for both men and women (n = 400). Genotyping was performed using the Illumina HumanOmni2.5™ BeadChip. Candidate SNPs were chosen based on the meta-analysis done by Olden et al. (Olden M. et al. 2014) where rs12917707 located near *UMOD* on chromosome 16 had the strongest association with urinary uromodulin levels. The rs4293393 variant located in the promoter region is in perfect linkage disequilibrium with rs12917707 in the HapMap CEU and shows a frequency within the CARTaGENE identical to HapMap CEU (International HapMap consortium 2005). The SNP rs12446492 in the adjacent gene *PDILT* (protein disulfide isomerase-like, testis-expressed) also independently influenced uromodulin levels (Olden M. et al. 2014). These 2 variants, partly in linkage disequilibrium (D' = 0.674) in the Canadian cohort, were included as genetic predictors of uromodulin excretion.

All studied parameters not normally distributed were log transformed. Pearson's or Spearman's correlation tests were used to check for correlations, t-test or Mann-Whitney U tests were used to

check for variance, multivariate linear regression was used to study associations between uromodulin and urinary parameters.

Results

Cohort	**n**	**Women** %	**Age** yr	**BMI** kg/m^2	**eGFR** ml/min per 1.73 m^2	**Urine uromodulin** µg/ml	**Urine uromodulin** mg/g creatinine
Colaus	5706	53	53 ± 11	25.7 ± 5	86 ± 15	25.7 (14.5 - 39.9)	18.2 (10.8 - 27.8)
SKIPOGH	817	53	45 ± 17	24.5 ± 4	98 ± 17	26.9 (17.4 - 34.9)	30.5 (19.2 - 44.0)
CARTaGENE	946	51	54 ± 9	26.7 ± 3	90 ± 14	35.2 (11.3 - 42.1)	14.2 (7.3 - 28 .2)

Table 1: Baseline characteristics of the participants in the studied cohorts. Values are expressed as mean ± SEM, median (25th - 75th percentiles) or as percentage.

Uromodulin and gender

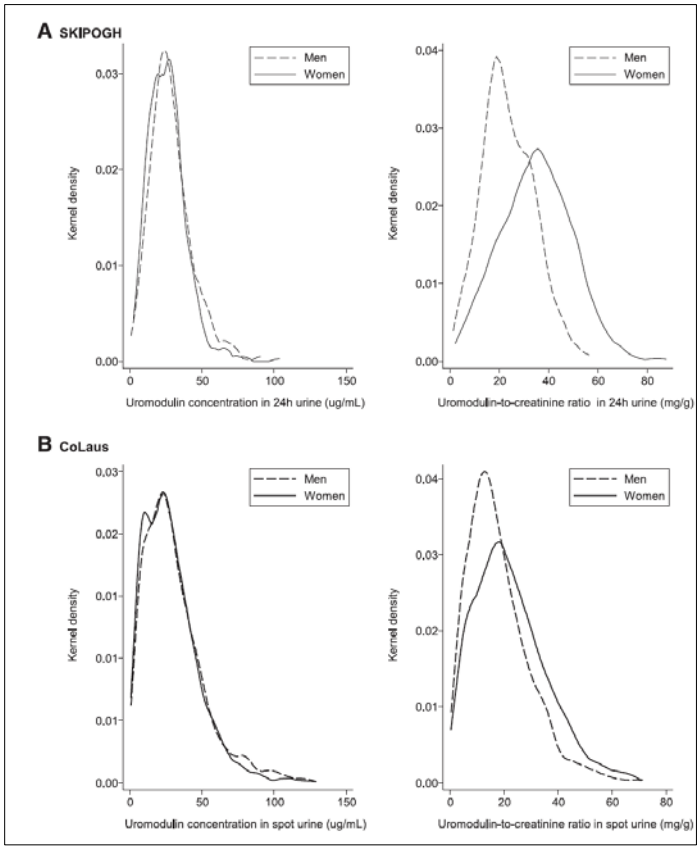

Figure 1: Distribution of uromodulin excretion (24 h urine) and concentration (spot urine) according to gender.

There were no gender dissimilarities in uromodulin concentration (in CoLaus, spot urine) nor in 24 h uromodulin excretion (SKPOGH). Differences observed in uromodulin to creatinine ratio are due to lower creatinine values in women (Figure 1). Since creatinine levels are body mass- and age-dependent adjusting for creatinine can cause an overcorrection.

Uromodulin and markers of glomerular filtration

Association between uromodulin excretion and eGFR were significantly positive in the assessed cohorts. In the Canadian cohort uromodulin excretion paralleled the eGFR (Figure 2 A). This has also been confirmed in both Swiss cohorts whether with creatinine-based or cystatin-based eGFR except that this association was positive only when eGFR was < 90 ml/min per 1.73 m^2 (Figure 2B).

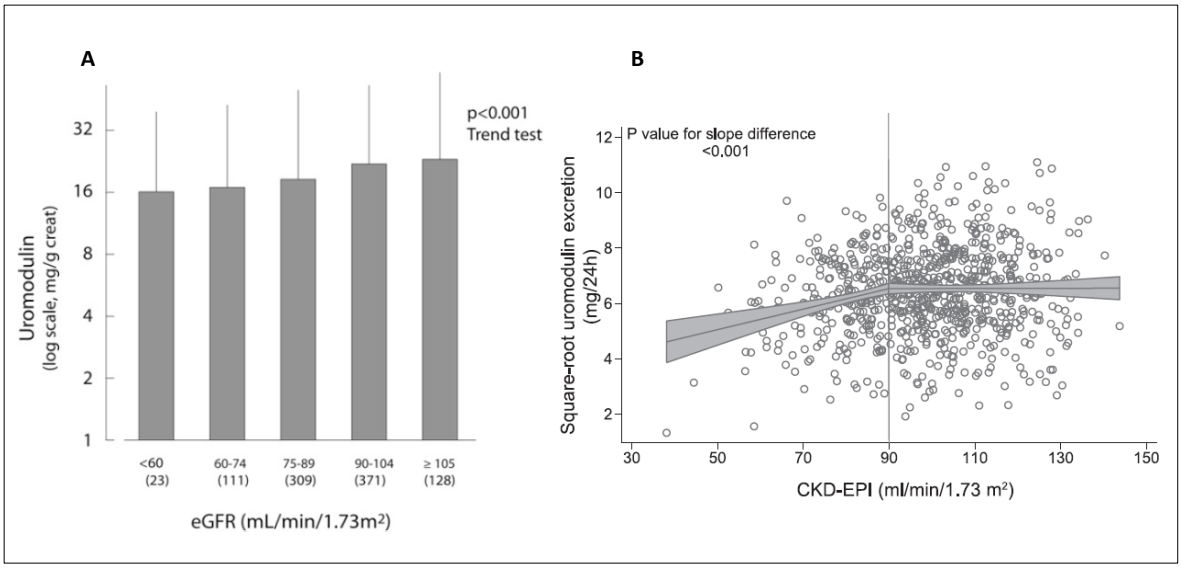

Figure 2: A) Association of eGFR with uromodulin excretion in CARTaGENE (P < 0.001) (error bars indicate SD). B) Scatterplot showing the association of eGFR with urinary uromodulin excretion in SKIPOGH.

Uromodulin and anatomic markers of renal mass

In SKIPOGH, renal grey-scale B-mode ultrasonography was performed. Study participants had their 24 h uromodulin excretion rate positively and linearly associated with renal length and volume in univariate and multivariate models (Figure 3).

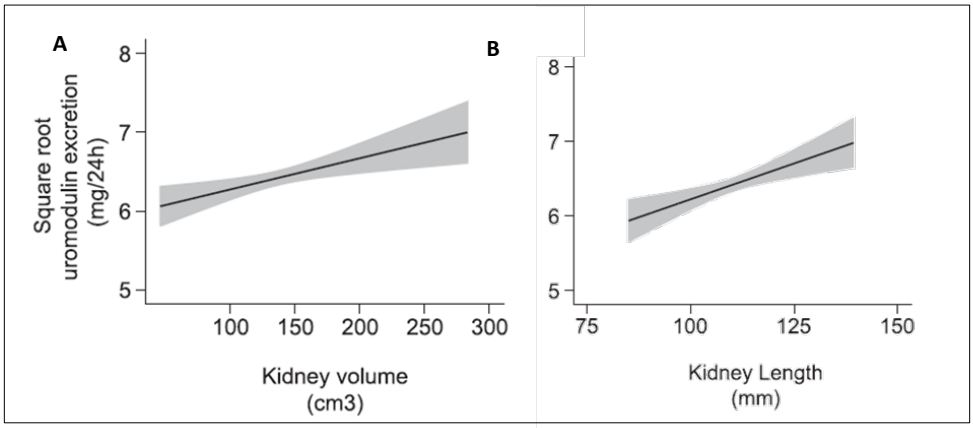

Figure 3: Age- and gender-adjusted associations of square rooted 24 h uromodulin excretion with kidney volume (A) and kidney length (B).

Genetic determinants of uromodulin excretion

Variants in the *UMOD* promoter region, such as rs4293393, are well documented to influence the excretion of urine uromodulin (Trudu M. et al. 2013). We confirm these findings in the Canadian cohort. Additionally, we find that SNP rs12446492 in the *PDILT* gene adjacent to the *UMOD* gene is predictive of uromodulin excretion. TT genotypes from both SNPs show highest levels of uromodulin (Figure 4).

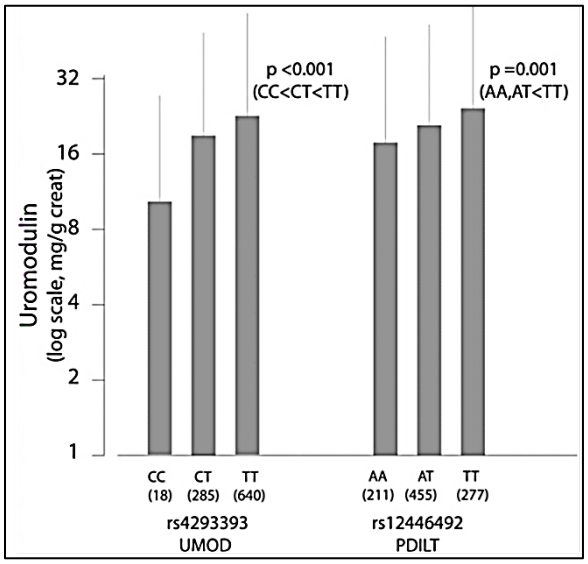

Figure 4: Associations of rs4293393 and rs12446492 genotypes with uromodulin excretion (Error bars indicate SD).

Urinary parameters and uromodulin excretion

We assessed the association of uromodulin levels with urine electrolytes, uric acid and glycosuria. In the Canadian cohort this association was inversely correlated with the presence of glycosuria. Glycosuria levels we considered positive if > 0.5 mg/g creat. (Santer R. et al. 2010).

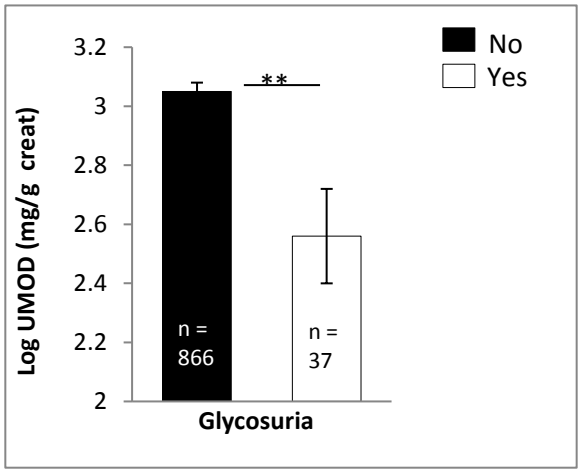

Figure 5: Variations of uromodulin levels among glycosuria categories. Uromodulin levels consistently decreased in the presence on glycosuria (error bars indicate SEM).

Univariate analysis showed strongly significant associations between uromodulin and electrolytes (Na^{2+}, K^{+}, Cl^{-}, Mg^{2+}, Ca^{2+}), fractional excretions of uric acid (FE-UA) sodium (FE-Na) and urine osmolality. Our results were consistent in the studied cohorts. These associations persisted when multivariate analysis was applied correcting for age, gender, BMI and eGFR. Some associations were weakened or lost when correction for creatinine was included (Table 2).

Variable	Unadjusted		Model 1		Model 2	
	Coefficient (95 % CI)	*P* Value	Coefficient (95 % CI)	*P* Value	Coefficient (95 % CI)	*P* Value
SKPOIGH (24 h urine excretion)						
Urinary sodium	2.07 (1.60 to 2.54)	<0.001	1.26 (0.81 to 1.72)	<0.001	0.98 (0.53 to 1.43)	<0.001
Urinary chloride	2.16 (1.52 to 2.79)	<0.001	1.22 (0.61 to 1.83)	<0.001	0.90 (0.29 to 1.50)	0.004
Urinary potassium	1.10 (0.74 to 1.47)	<0.001	0.43 (0.11 to 0.75)	0.01	0.07 (-0.23 to 0.37)	0.64
Urinary magnesium	0.24 (0.17 to 0.32)	<0.001	0.12 (0.05 to 0.20)	0.001	0.06 (-0.01 to 0.13)	0.09
Urinary calcium	0.38 (0.24 to 0.52)	<0.001	0.23 (0.09 to 0.4)	0.002	0.16 (0.02 to 0.30)	0.03
Urinary osmolality	0.82 (0.63 to 1.01)	<0.001	0.54 (0.37 to 0.70)	<0.001	0.33 (0.18 to 0.48)	<0.001
CoLaus (Spot urine)						
Urinary sodium	1.87 (1.40 to 2.34)	<0.001	1.59 (1.14 to 2.04)	<0.001	1.63 (1.14 to 2.11)	<0.001
Urinary chloride	3.88 (3.34 to 4.42)	<0.001	3.45 (2.93 to 3.98)	<0.001	2.38 (1.82 to 2.94)	<0.001
Urinary potassium	6.40 (5.90 to 6.91)	<0.001	6.14 (5.64 to 6.65)	<0.001	2.63 (2.15 to 3.12)	<0.001
Urinary magnesium	0.81 (0.71 to 0.9)	<0.001	0.77 (0.67 to 0.86)	<0.001	0.02 (-0.07 to 0.10)	0.72
Urinary calcium	0.59 (0.45 to 0.73)	<0.001	0.54 (0.40 to 0.68)	<0.001	0.09 (-0.06 to 0.23)	0.25
Urinary osmolality	0.74 (0.69 to 0.79)	<0.001	0.68 (0.63 to 0.73)	<0.001	0.24 (0.20 to 0.29)	<0.001

Table 2: Association of urinary osmolality and electrolytes with urinary uromodulin in Swiss cohorts. Model 1: adjusted for age, sex, body weight, eGFR and 24 h urine volume (SKIPOGH only). Model 2: adjusted as for model 1 plus urinary creatinine concentration in CoLaus and 24 h creatinine excretion in SKIPOGH. 95 % CI: 95 % confidence interval.

To check for independent factors associated with uromodulin excretion we carried out in the CARTaGENE population a stepwise multivariate linear regression including variables that showed significance in univariate analysis. Higher uromodulin levels were independently associated with eGFR, TT genotypes from rs4293393 and rs12446492, FE-Na and FE-UA. On the other hand, glycosuria was associated with lower levels of uromodulin excretion. FE-UA explained most of the variability in the model (Table 3).

Variables	Standardized-ß	*P* Value
GFR CKD-EPI (ml/min per 1.73 m^2)	0.11	0.001
rs4293393 (TT vs CC vs CT)	0.07	0.04
rs12446492 (TT vs AA vs AT)	0.09	< 0.01
FE-Na	0.1	< 0.01
Presence of glycosuria	-0.07	0.02
FE-UA	0.29	< 0.01

Table 3: Multivariate predictors of uromodulin in CARTaGENE.

Discussion

In this study, we evaluate determinants of uromodulin excretion in three large cross-sectional studies. We show a correlation between uromodulin excretion and several clinical, genetic and urinary variables. Urine uromodulin excretion is positively associated with renal mass, renal volume, eGFR, urine electrolytes and osmolality suggesting that urinary uromodulin can be considered as a marker for tubular functions and mass. Conversely, we show that uromodulin excretion decreases with age and in the presence of glycosuria. We also confirm that urinary uromodulin levels are influenced by genetic variants located in the *UMOD* promoter and the *PDILT* gene.

The association of uromodulin with renal volume and renal mass suggests that uromodulin can be considered as a marker for tubular mass reflecting tubular number. Also, the validation of a positive relationship between uromodulin and eGFR on one hand and the decline of uromodulin excretion with age on the other hand, imply that uromodulin reveals tubular activity and that the amount of functional tubules can be associated with uromodulin excretion levels.

A decrease in uromodulin excretion is documented in the presence of diabetes mellitus indicators such as glycosuria while eGFR levels remain normal. Diabetes might be inducing a toxic effect on renal tubular cells leading to an underlying early tubular dysfunction independent of eGFR. These findings are coherent with a study performed on CKD patients with diabetes but without glomerular dysfunction (Möllsten A. et al. 2010). Confirmation of these results is needed in a large diabetic-specific study. Moreover, it has been shown that glycosuria can affect the TAL activity thus modulating uromodulin excretion (Riazi S. et al. 2006).

In the studied cohorts, changes in uromodulin levels correlate positively with changes in urinary electrolytes and urine osmolality. Uromodulin excretion (spot urine and 24h urine) parallels sodium, potassium, chloride, calcium and magnesium urinary excretion, indicating that uromodulin production modifies tubular handling of these electrolytes and reflects tubular function. Despite the robustness of these associations, the dietary effect on sodium, chloride and potassium excretion and tubular handling should not be ignored unlike calcium excretion, which is less affected by the dietary intake hence reflecting the TAL function more closely. Urine osmolality reveals tubular activity, therefore its positive association with uromodulin supports the above-mentioned conclusion of uromodulin being a good indicator of tubular activity. These findings are in line with results from *in vitro* and *in vivo* studies showing uromodulin, as the regulator of transport processes in the TAL through increasing the activity of ROMK and enhancing the phosphorylation of NKCC2, affects urine concentrating ability (Renigunta A. et al. 2011; Mutig K. et al. 2011). The highly significant positive correlation between fractional excretion of uric acid and uromodulin could be explained by volume expansion in the proximal tubule: an increase in uromodulin excretion would enhance NKCC2 phosphorylation in the TAL (Mutig K. et al. 2011) which is hypothesized to cause volume expansion in the proximal tubule resulting in declined reabsorption of sodium and uric acid thus a rise in uric acid fractional excretion (Scolari F. et al. 2004). These results show that despite the exclusive expression of uromodulin in the TAL, a dysfunction in this tubule might affect the function of the adjacent proximal tubule.

This work identifies factors influencing and modulating the excretion of uromodulin in the general population. Moreover, it suggests uromodulin as a biomarker for renal tubular function in the general population as well as a marker for tubular damage independent of eGFR.

The strength of this work is the large sample size with detailed information about participants; the centralized measurements of uromodulin and urinary parameters using a well-established method, measurement of renal length and renal mass using a standardized protocol whereas the limitations are the lack of ethnic diversity and the limitation in assessing causal conclusions because these are cross-sectional studies.

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VI. Validation of surrogates of urine osmolality in population studies

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Abstract

The importance of vasopressin and/or urine concentration in various kidney, cardiovascular and metabolic diseases has been emphasized recently. Due to technical constraints, urine osmolality (U_{osm}), a direct reflect of urinary concentrating activity, is rarely measured in epidemiologic studies.We analyzed two possible surrogates of U_{osm} in 4 large population-based cohorts (total n = 4,247) and in patients with chronic kidney disease (CKD, n = 146). An estimated U_{osm} (eU_{osm}) based on the concentrations of sodium, potassium and urea, and a urine concentrating index (UCI) based on the ratio of creatinine concentrations in urine and plasma were compared to the measured U_{osm} (mU_{osm}).

eU_{osm} is an excellent surrogate of mU_{osm}, with a highly significant linear relationship and values within 5 % of mU_{osm} (r = 0.99 or 0.98 in each population cohort). Bland-Altman plots show a good agreement between eU_{osm} and mU_{osm} with mean differences between the two variables within ± 24 mmol/L. This was verified in men and women, in day and night samples, and in CKD patients. The relationship of UCI with mU_{osm} is also significant but is not linear and exhibits more dispersed values. Moreover, the latter index is no longer representative of mU_{osm} in patients with CKD as it declines much more quickly with declining GFR than mU_{osm}.

The eU_{osm} is a valid marker of urine concentration in population-based and CKD cohorts. The UCI can provide an estimate of urine concentration when no other measurement is available but should be used only in subjects with normal renal function.

Introduction

The interest in the influence of the antidiuretic hormone vasopressin (ADH or AVP) as a significant player in various kidney, cardiovascular and metabolic diseases has been revived recently (Torres VE. 2009; Bolignano D. et al. 2010; Cirillo M. et al. 2010; Devuyst O. et al. 2013). The availability of non-peptide, orally active selective vasopressin receptor antagonists (vaptans) (Greenberg A. et al. 2006; Decaux G. et al. 2008) and of a reliable ELISA for the measurement of copeptin, a validated surrogate of vasopressin (Morgenthaler NG. 2010; Roussel R. et al. 2014), has opened the door for a number of studies addressing the vasopressin/thirst pathway and osmoregulation in general (Bankir L. 2013).

Independent of the well-known contribution of ADH to various forms of water disorders, recent epidemiological studies have shown significant associations between indices of the vasopressin/hydration system and the incidence or progression of diseases including chronic kidney disease (CKD), autosomal dominant polycystic kidney disease (ADPKD), diabetic nephropathy, obesity, metabolic syndrome, and insulin resistance (Bardoux P. et al. 2003; Meijer E. et al. 2010; Enhorning S. et al. 2011; Roussel R. et al. 2011; Ho TA. et al. 2013; Devuyst O. et al. 2013; Schoen T. et al. 2013; Velho G. et al. 2013; Ong AC. et al. 2015; Ponte B. et al. 2015; Roussel R. et al. 2015; Pruijm M. et al. 2016; Roussel R. et al. 2016). A number of experimental studies have demonstrated the adverse effects of vasopressin or a low level of hydration in animal models of these disorders (Bouby N. et al. 1990; Bardoux P. et al. 2003; Ahrabi AK. et al. 2007; Perico N. et al. 2009; Koshimizu TA. et al. 2012; Taveau C. et al. 2015). A recent double-blind, placebo-controlled clinical trial, using a selective vasopressin V2 receptor antagonist, tolvaptan, proved to bring significant benefit over a 3 year period in patients with ADPKD and well preserved renal function (Torres VE. et al. 2012).

Because AVP is difficult to measure due to its small mass, very low circulating concentrations, poor stability *in vitro*, and time-consuming assay, most of the recent studies dealing with this hormone rely on the measurement of copeptin (a peptide that is part of the pre-pro-hormone containing vasopressin) in plasma or, more indirectly, on fluid intake or daily urine volume (Clark WF. et al. 2011; Strippoli GF. et al. 2011). Urine osmolarity (U_{osm}), the most direct parameter reflecting the action of AVP on distal tubular segments of the kidney, is rarely measured due to technical constraints and is thus usually not available in epidemiologic studies.

Various surrogates of U_{osm} have been used in clinical studies. They include the specific urine density or the refraction index that give only an approximate value of the solute content in the urine and are subject to several biases including distortion in the case of proteinuria and poor precision of readings. Two other surrogates are the Urine Concentrating Index (UCI) based on the handling of creatinine by the kidney (Bankir L. et al. 2007; Perucca J. et al. 2007), and the estimated urine osmolarity (eU_{osm}) based on the concentration of the three main osmoles present in the urine: sodium, potassium and urea (Perucca J. et al. 2007; Zittema D. et al. 2014). To our knowledge, the validity of these two surrogates has not been evaluated in large, population-based cohorts with normal or altered renal function. The aim of the present study was to assess the value of eU_{osm} and UCI compared to measured U_{osm} (mU_{osm}) in large population-based and CKD cohorts, and to test the influence of sample type, gender and age on these markers.

Material and Methods

Cohorts

The general characteristics of the subjects belonging to the different cohorts are presented in Table 1.

Cohort	CROATIA-Korcula	GS:SFHS Aberdeen	GS:SFHS Glasgow	SKIPOGH Day	SKIPOGH Night	CKD Necker
N	463	554	2305	925	idem	146
Sample type	Spot	Spot	Spot	Day period	Night period	24 h
Age, y	58 (19 - 87)	57 (19 - 88)	53 (18 - 93)	47 (18 - 90)	idem	64 (17 - 86)
Gender : M / W %	41 / 59	43 / 57	41 / 59	47 / 53	idem	59 / 41
BMI	27.97 ± 0.21	27.22 ± 0.22	26.97 ± 0.21	25.03 ± 0.15	idem	24.16 ± 0.30
mU_{osm}, mosm/kg H_2O	668 ± 10	524 ± 10	540 ± 5	457 (110 - 1174)	541 (67 - 1304)	396 ± 13
eU_{osm}, mosm/L	664 ± 9	526 ±11	547 ±5	450 (118 - 1142)	513 (61 - 1223)	381 ± 12
UCI	165 ± 4	119 ± 3	117 ± 2	114 ± 2	145 ± 3	41.6 ± 3.6
U_{urea}, mmol/L	285 ± 5	225 ± 5	250 ± 3	227 ± 4	296 ± 5	177 ± 6
U_{Na}, mmol/L	113.8 ± 2.2	79.3 ± 2.0	83.9 ± 1.0	94.8 ± 1.6	93.4 ± 1.6	68.7 ± 2.8
U_K, mmol/L	65.9 ± 1.6	63.5 ± 1.5	64.9 ± 0.8	47.4 ± 0.8	32.7 ± 0.6	33.3 ± 1.3
eGFR . ml/min/1.73 m^2	83.4 ± 1.1	92.2 ± 0.7	89.1 ± 0.4	96.3 ± 0.6	—	46.2 ± 2.5

Table 1: Demographic information about the different cohorts. Means + SEM or median (interval).

1. Generation Scotland: SFHS (GS: SFHS) and CROATIA-Korcula

Aberdeen and Glasgow subjects were selected from the Generation Scotland study, a family-based genetic epidemiology study that included 24,000 volunteers from across Scotland, as previously described (Smith BH. et al. 2006). Biological samples including morning spot urine, were collected during participation from 2006-2011 (Olden M. et al. 2014). We also studied subjects from the CROATIA-Korcula cohort, a family-based, cross-sectional study from the island of Korcula (Croatia) that initially included 965 subjects, as previously described (Polasek O. et al. 2009). Studies of these three cohorts included clinical information, biochemical measurements, and lifestyle and health questionnaires. For the present study, subjects from these three cohorts were randomly selected for measurement of U_{osm} (n = 554 from GS: SFHS Aberdeen, 2,305 from GS: SFHS Glasgow and 463 from CROATIA-Korcula). All participants provided written informed consent. For GS: SFHS national ethical approval has been obtained from the National Health Service Tayside Research Ethics committee. The CROATIA-Korcula study was approved by the Ethical Committee of the Medical School, University of Zagreb.

2. SKIPOGH

SKIPOGH (Swiss Kidney Project on Genes in Hypertension) is a family- and population-based cross-sectional multi-center study that examines the genetic determinants of blood pressure. Participants were recruited in 2009-2013 in the cantons of Bern and Geneva, and the city of Lausanne. Detailed methods have been previously described. (Pruijm M. et al. 2013; Ponte B. et al. 2014). The study visit was performed in the morning after an overnight fast. Participants were asked to bring urine of the previous 24 h collected separately during day and night periods defined according to each participant's self-reported bedtime and wake-up time. The SKIPOGH study was approved by the Human Research Ethics Committee of Lausanne University Hospital and University of Lausanne (Lausanne, Switzerland), by the Ethics Committee for the Research on Human Beings of Geneva University Hospitals (Geneva, Switzerland), and by the Ethics Committee of the Canton of Bern, (Bern, Switzerland).

3. CKD patients

This study includes 146 out-patients with CKD of diverse etiologies and various levels of renal dysfunction, attending the Nephrology Department of Necker Hospital (Paris, France) in

1993 for a bi-annual checkup (Jungers P. 1995; Roussel R. et al. 2015). All patients provided 24 h urine. Informed consent was obtained for storage of the samples and additional future measurements to enable a more complete understanding of the pathophysiological characteristics related to CKD. On the freshly collected plasma and urine samples, osmolality was measured with a freezing point osmometer (Roebling, Berlin, Germany). Creatinine concentration was measured by the Jaffe colorimetric method and creatinine clearance in ml per 1.73 m^2 was used as an estimate of GFR. Concentration of urinary solutes was measured with a classical automatic multi-analyzer.

Measurements in Plasma and Urine Samples

In the 4 population-based cohorts, urine samples were kept frozen at -80 °C until measurements of U_{osm} and urinary solute concentrations. Sodium, potassium, glucose, creatinine, and urea were measured with a Beckman Coulter Synchron System Assays (Unicell DxC Synchron Clinical System). The CKD-EPI formula was used to calculate eGFR (van den Brand JA. et al. 2011) U_{osm} was measured on 20 µl samples by the freezing-point depression technique using an Advanced Osmometer (Massachusetts 02062, USA). A control (Clinitrol 290) and a set of calibration standards (50, 850 and 2000 mosm/kg H_2O) were used before running each batch. The intra-assay coefficient of variability was 0.19 % and the inter-assay coefficient of variability was 1.32 %.

Calculations and Statistical Analyses

Most modern osmometers measure the osmolality of the fluids in milliosmoles per kg water (mosm/kg H_2O) (see footnote 1). Osmolarity expresses the concentration of osmotically active molecules in milliosmoles per liter of water (mosm/L). Sweeny and Beuchat described the technical aspects and limitations of osmometry methods and provided detailed considerations about the concepts of osmotic pressure, osmolarity, osmolality and solute concentrations (Sweeney TE. et al. 1993).

Footnote 1: The terms osmolarity or osmolality should be preferred to "osmotic pressure" because this physical osmotic force is not a pressure. It was named in this way in the past, when osmolarity was evaluated indirectly as a hydrostatic pressure generated by an unknown fluid opposed to a reference fluid, separated by a semi-permeable membrane. The measurements were expressed in mm height between the levels of the two fluids in the two compartments.

Estimated urine osmolarity (eU_{osm})

The major urinary solutes, accounting for more than 90 % of all urinary osmoles, are urea and the two cations sodium and potassium along with their accompanying anions. Thus, their cumulated concentrations (in mmol/L) should be close to the actual U_{osm} (in mosm/L). An "estimated" U_{osm} (eU_{osm}) can be calculated according to the following formula:

$$eU_{osm} = (U_{Na} + U_K)*2 + U_{urea}$$

Where U_{Na}, U_K and U_{urea} are the urinary concentrations of sodium, potassium and urea respectively, in mmol/L. (U_{Na} + U_K) is multiplied by 2 to account for the accompanying anions. If urea was measured as urea nitrogen, it should be remembered that there are two atoms of nitrogen (MW = 14) per molecule of urea. Urea in mmol/L = urea nitrogen in mg/dL x 0.357 (explanation: urea nitrogen in mg/dL multiplied by 10 (conversion of dL to L) and divided by 14x2 (mg N per mmol urea). In case of significant glycosuria, glucose concentration can be added to the formula.

Urine Concentrating Index (UCI)

Creatinine is freely filtered and is assumed to undergo negligible secretion or reabsorption along the nephron when kidney function is normal. Thus, the concentration of creatinine in urine relative to that in plasma (U_{creat} and P_{creat}, respectively), i.e., the ratio of urine-to-plasma creatinine concentrations, is proportional to the fraction of filtered water that has been reabsorbed to concentrate the solutes in the urine. This ratio provides an Index of Urine Concentration (UCI), a ratio that has no unit:

$$UCI = U_{creat} / P_{creat}$$

Statistical analyses

The Statistical Package for Social Sciences (SPSS) version 19 (IBM Corporation, New York) and GraphPad Prism 5 were used to carry out the statistical analyses and generate the figures. Results are shown as means ± SEM for normally distributed variables, or as medians and 25 % - 75 % interquartile range (IQR) for other variables. The agreement between mU_{osm} and eU_{osm} was assessed by Bland-Altman plots. The Shapiro-Wilk test was used to assess the distribution of mU_{osm} in the SKIPOGH study. Correlations were studied using Pearson's correlation analysis (in case of normality) or Spearman’s rho test (for other variables). Wilcoxon

signed-rank test was used to compare repeated measures for day and night urine samples of the SKIPOGH subjects. The significance level was set at 5 %.

Results

U_{osm} surrogates in population-based cohorts

Large variations in urine concentration are observed among individuals. The mU_{osm} in different subjects varies from ≈ 150 to 1,200 mosm/kg H_2O in spot urine of the three population-based cohorts as well as in day and night urine of the SKIPOGH cohort (Figures 1A and 2B). A substantial number of subjects (21 %) dilute their urine below plasma osmolality whereas others (9 %) concentrate their urine up to three times more than the level of plasma osmolality (Figure 2A). These extreme mU_{osm} are not associated with differences in eGFR.

Highly significant linear correlations are observed between mU_{osm} and eU_{osm} in all populations (CROATIA-Korcula r = 0.98, GS: SFHS Aberdeen r = 0.98, GS: SFHS Glasgow r = 0.99) (Figures 1A and 2B). The best fit linear regression lines are almost superimposed with the medians.

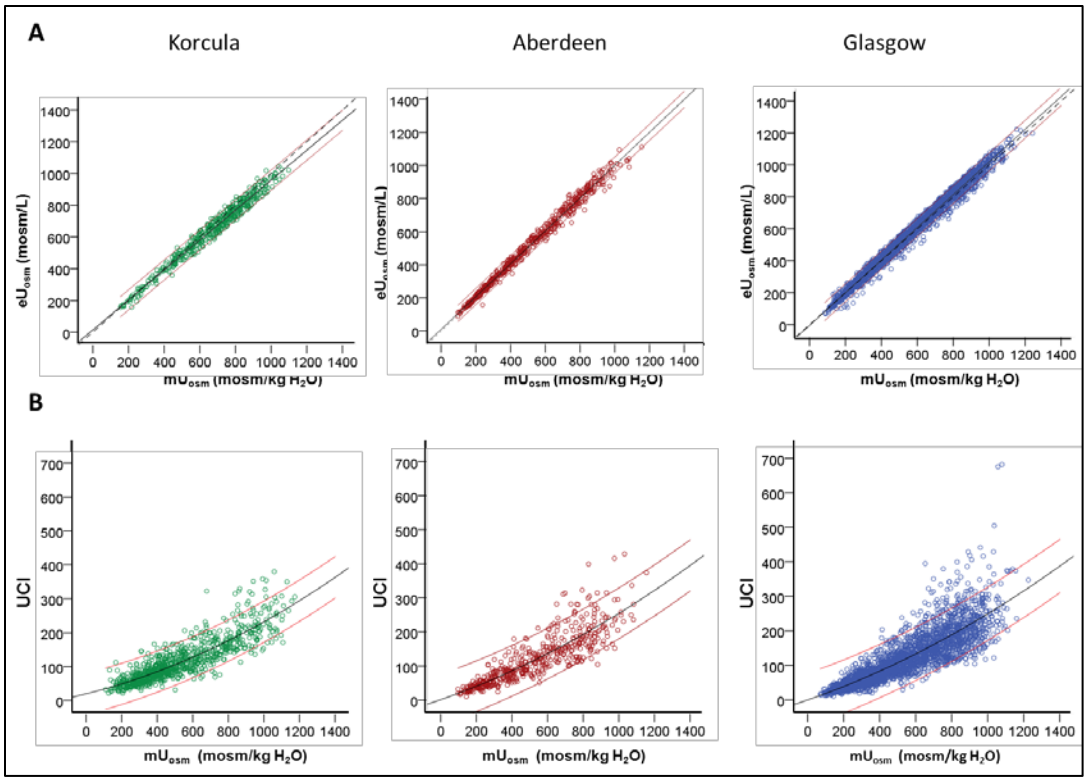

Figure 1: Comparison of urine osmolality surrogates with measured urine osmolality. A:. Linear correlation between measured osmolality and estimated osmolarity in 3 cohorts. CROATIA-Korcula: mUosm = 1.03 eUosm + 3.3 ($p < 0.001$, $r = 0.98$); GS:SFHS Aberdeen: mUosm = 0.99 eUosm – 4.4 ($p < 0.001$, $r = 0.98$); GS:SFHS Glasgow: mUosm = 0.96 eUosm + 11 ($p < 0.001$, $r = 0.99$). The thin vertical lines show mUosm of 300 and 900 mosm/kg H2O, i.e. approximatively one time and three times the plasma osmolality. B:. Quadratic correlation between UCI and measured urine osmolality in 3 cohorts. CROATIA-Korcula: mUosm = 5.04 UCI – 0.009 UCI2 + 126 ($p < 0.001$, $r = 0.76$); GS:SFHS Aberdeen: mUosm = 5.52 UCI – 0.009 UCI2 + 28 ($p < 0.001$, $r = 0.90$); GS:SFHS Glasgow: mUosm = 4.89 UCI – 0.006 UCI2 + 90 ($p < 0.001$, $r = 0.89$). Black lines represent the best-fit curves. Red thin lines represent the 95 % confidence intervals. Dotted lines in the top panel represent the medians.

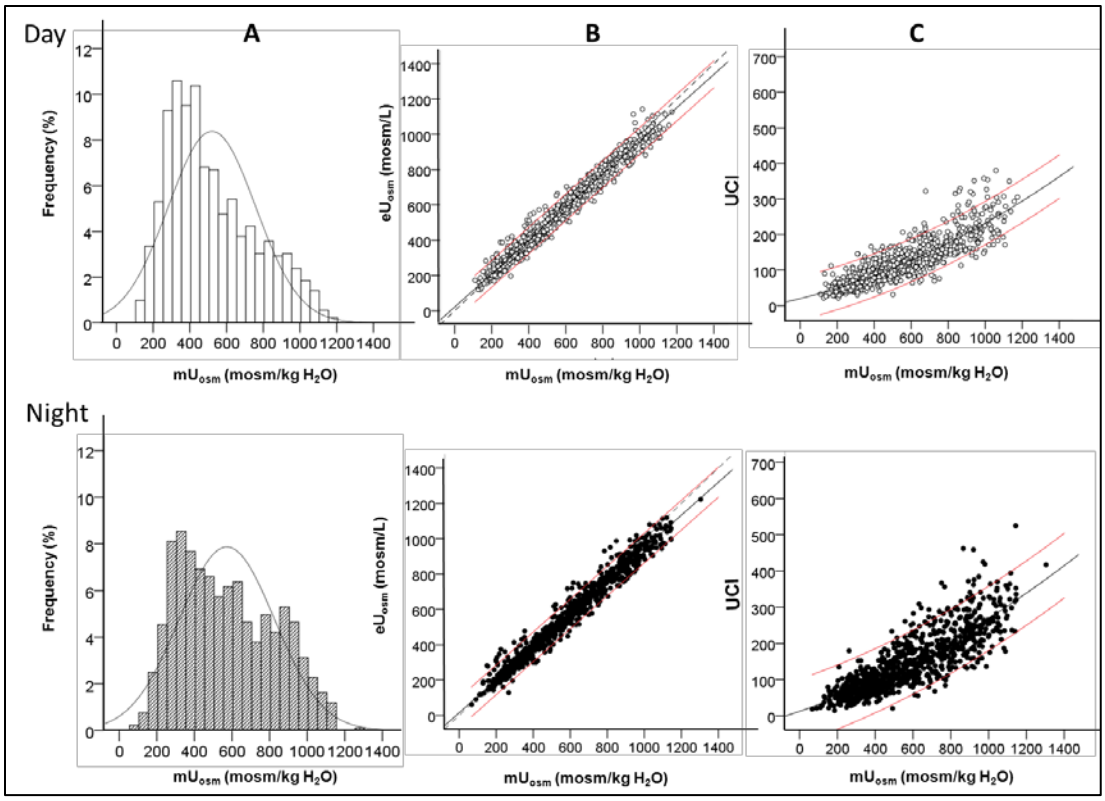

Figure 2: Day-time and night-time urine in the SKIPOGH population (n = 925). A: Distribution of mUosm among SKIPOGH subjects. Thin curves represent the normal distribution model. B: Linear correlation between measured and estimated Uosm in daytime and night-time urine. C: Quadratic correlation between UCI and mUosm in daytime versus night-time urine. In B and C, black lines represent the best-fit curves and red thin lines 95 % confidence intervals. Dotted lines in B represent the medians.

Bland-Altman plots show a good agreement between eU_{osm} and mU_{osm} in the three population based cohorts (Table 1). This is reflected in the small bias values (CROATIA-Korcula bias = 24, GS: SFHS Aberdeen bias = -6, GS: SFHS Glasgow bias = -23), and relatively narrow precision range (CROATIA-Korcula -44 to 90, GS: SFHS Aberdeen -54 to 43, GS: SFHS Glasgow -80 to 34). Plot for the GS: SFHS Aberdeen population is given as an example in Figure 3.

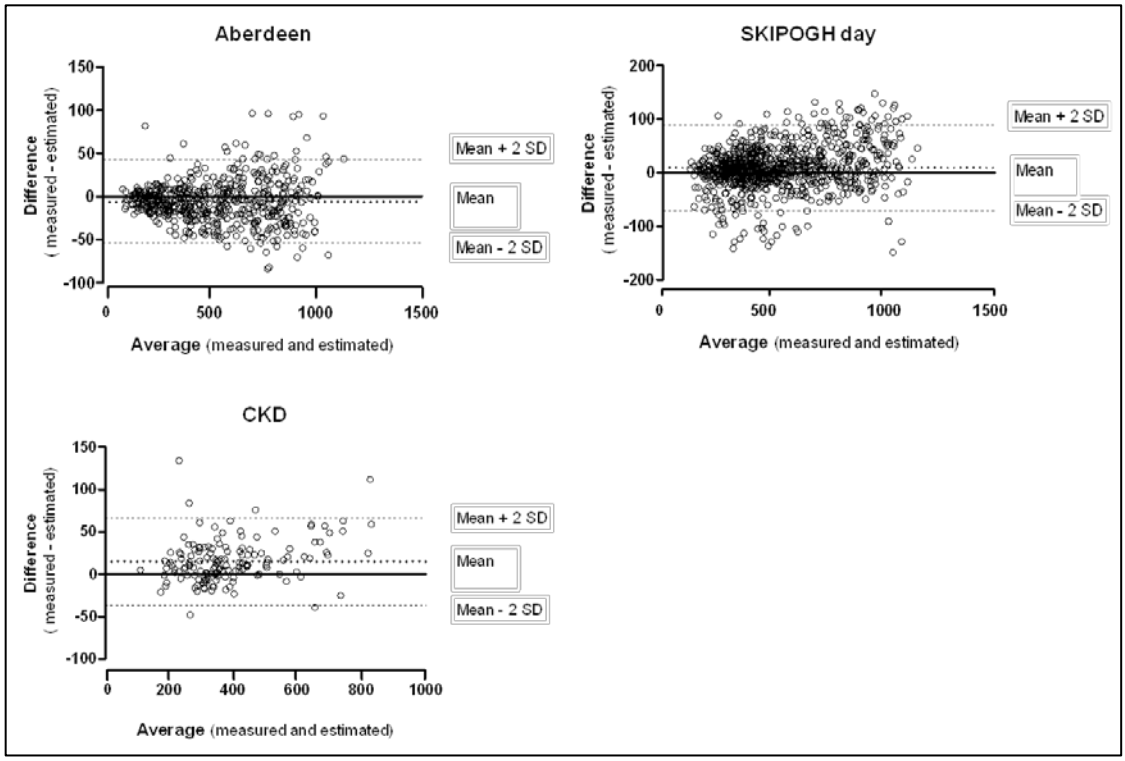

Figure 3: Bland-Altman plots showing the agreement between estimated and measured Uosm in the spot urine samples of GS:SFHS Aberdeen (top), the day urine samples of SKIPOGH (middle) and the 24 h urine of the CKD patients.

Although the relations between UCI and mU_{osm} are significant, they exhibit a relatively large dispersion of individual values, increasing with increasing osmolality (Figures 1B and 2C). Nonetheless, as an average, the ratio of UCI to mU_{osm} is fairly constant (0.20, 0.21 and 0.22 for mU_{osm} = 300, 600 and 900 mosm/kg H_2O, respectively). UCI can be approximately converted to osmolality by the following quadratic formula: $mU_{osm} = 5\ UCI - 0.007\ UCI^2 + 83$.

The possible influence of glycosuria that occurred in some subjects on eU_{osm} was evaluated. Among 3,322 subjects of the three cohorts in which urinary glucose was available, 58 exhibited glucosuria > 1.66 mmol/L [41] (mean ± SEM 11.58 ± 2.28 mmol/L; range 1.7 to 86.8). Their age and eGFR were 57.0 ± 1.8 y and 86.8 ± 2.4 ml/min, 1.73 m^2, respectively. Measured U_{osm} in these subjects was 642 ± 32 mosm/kg H_2O. Estimated U_{osm}, calculated without or with the addition of urinary glucose was 624 ± 31 and 635 ± 32 mosm/L respectively, both within 3 % of mU_{osm}.

Urine osmolality is known to be higher in men than in women. This was verified in the cohorts of the present study (Table 2**)**: men exhibited higher mU_{osm} and eU_{osm} than women although the magnitude of this gender difference differed among the three populations. eU_{osm} was very close to mU_{osm} in both genders and the men/women ratio of eU_{osm} was very similar to that of mU_{osm}. For UCI, there was a tendency for more inter-individual variation in women than in men as well as lower men/women ratios which tended to underestimate the gender difference.

U_{osm} surrogates in day and night urine

In healthy subjects, urine is usually more concentrated during the night than during the day. We investigated if the relationships between mU_{osm}, eU_{osm} and UCI are comparable in day and night urine of the 925 subjects of the SKIPOGH study (Figure 2A). The Shapiro-Wilk test indicates that these variables diverge from a normal distribution (shown by a thin curve). Mean mU_{osm} ± SEM during day and night was respectively 520 ± 4 and 572 ± 7 mosm/kg H_2O. Median (IQR) values were 457 (334 – 676) and 541 [356 - 777] mosm/kg H_2O, respectively ($p < 0.001$, Wilcoxon signed-rank test). The histograms of mU_{osm} during day and night do not follow a normal distribution and there is a tendency for a bimodal distribution during the night.

Measured and estimated U_{osm} values exhibit highly significant linear correlations in both day and night urine (Figure 2B), as also observed in the spot urine of the other cohorts. Bland-Altman plots show a good agreement between eU_{osm} and mU_{osm} in day and night urine, as reflected by the small bias values (day bias = 9, night bias = 24), and the precision range (day -71 to 89, night -66 to 114) (Figure 3). The relations between UCI and mU_{osm} are best described by quadratic correlations. Thin red lines show the 95 % confidence intervals. As in the three cohorts shown in Figure 1, UCI vs mU_{osm} values were more widely dispersed than eU_{osm} vs mU_{osm} values.

Uosm surrogates in CKD patients

Table 2 compares the values of eU_{osm} and UCI to those of mU_{osm} in CKD patients, according to their level of renal function. In all CKD classes, eU_{osm} is very close to mU_{osm}. Both variables decline in parallel with declining eGFR. Bland-Altman plot show a relatively good agreement between the two methods, as reflected by the small bias value (15) and the precision range (37 to 67) (Figure 3). In contrast, UCI declines much more dramatically than mU_{osm}. These differences are due mostly to the progressive rise in plasma creatinine concentration (from 91 ± 5 to 514 ± 34 µmol/L in the two extreme classes, a 5.6 fold increase) while urine creatinine concentration declines only two-fold as a result of a lower total creatinine excretion and a moderately higher 24 h urine volume. In these patients, a spot urine sample was collected in the morning following the 24 h urine collection (Table 2). mU_{osm} in morning urine is 10 - 20 % higher than mU_{osm} in 24 h urine, a difference that seems independent of the level of renal function.

CKD Stage	**N**	**Creatinine Excretion** mmol/d	**Spot mU_{osm}** mosm/kg H_2O	**mU_{osm}** mosm/kg H_2O	**eU_{osm}** mosm/L	**eU_{osm} / mU_{osm}**	**UCI** (U_{creat} /P_{creat})	**UCI*100 / mU_{osm}** (1)
Stage 1 (> 90)	13	13.4 ± 1.0	738 ± 61	650 ± 48	608 ± 43	0.94 ± 0.01	146 ± 17	22.1 ± 1.6
Stage 2 (60 - 89)	29	13.2 ± 0.7	517 ± 32	479 ± 34	458 ± 34	0.95 ± 0.01	59 ± 5	12.3 ± 0.4
Stage 3 (30 - 59)	54	11.5 ± 0.5	450 ± 14	371 ± 14	358 ± 14	0.97 ± 0.01	34 ± 2	9.2 ± 0.3
Stage 4 (15 - 29)	32	9.4 ± 0.5	376 ± 13	321 ± 13	311 ± 12	0.98 ± 0.01	17 ± 1	5.2 ± 0.3
Stage 5 (< 15)	19	8.1 ± 0.5	318 ± 11	296 ± 12	291 ± 12	0.98 ± 0.02	7 ± 1	2.5 ± 0.2

Table 2: Osmolality and its surrogates in 147 CKD patients according to the level of renal function
Means ± SEM. CKD stages are shown with the limits of eGFR in ml/min per 1.73 m^2.
Spot mUosm = mUosm of a morning spot urine sample. All other values concern 24 h urine collection.
(1) For the ratio of UCI/mUosm. UCI was multiplied by 100 to make the reading easier.

Discussion

The urine concentrating activity of the human kidney was rarely investigated, except in a few conditions such as urolithiasis and diabetes insipidus. Recent experimental and epidemiological findings have renewed the interest in the components of the water balance and in the parameters reflecting this integrative function (Bouby N. et al. 1990; Perico N. et al. 2009; Torres VE. 2009; Clark WF. et al. 2001; Strippoli GF. et al. 2011; Bankir L. et al. 2013; Devuyst O. et al. 2013; Perrier E. et al. 2013; Zittema D. et al. 2014; Perrier ET. et al. 2015; Ponte B. et al. 2015). It is indeed quite different for the kidney to excrete a daily osmolar load of 900 mosm in 1 L of urine at 900 mosm/L or in 3 L of urine at 300 mosm/L. Increased urine concentration (associated with increased solute-free water reabsorption) results in a lower fractional excretion of several solutes and in a significant hyperfiltration that is, at least in part, mediated by vasopressin acting on renal V2 receptors. It has been proposed that this hyperfiltration is mediated by changes in the composition of the tubular fluid at the macula densa, resulting from vasopressin's action on water, sodium and urea transport in the collecting duct and the resulting recycling of urea in the medulla (Bankir L. et al. 2013). U_{osm}, the most direct reflect of the urine concentrating activity, is rarely measured in large cohorts because of technical issues (see below). The present study, in a cross-sectional design, describes two practical, easily accessible surrogates of U_{osm} and assesses their validity by comparing the results to the actually measured U_{osm} in large cohorts of the population and in a group of patients with CKD. We also checked the value of these surrogates in various sample types (spot or 24 h, day and night), and according to gender and to renal function.

Our results show that the *estimated* U_{osm}, based on sodium, potassium and urea concentrations, is an excellent surrogate of the *measured* U_{osm}. In most cases, eU_{osm} is within ± 5 % of mU_{osm}. This is similarly true in men and women, as well as in urine collected during day or night, and in patients with impaired renal function at any level of GFR. One may wonder how eU_{osm} and mU_{osm} may be so close when the formula used for the calculation of eU_{osm} neglects the minor solutes that should, however, represent more than 5 % of all urinary solutes. This is partly explained by the fact that the units are not the same. eU_{osm} is expressed in mosm/L while mU_{osm} is in mosm/kg H_2O. Because one liter of water with dissolved solutes weights more than 1 kg, the osmolality is lower than the osmolarity. The two measures differ only modestly for solutions

within the biological range. For example, a solution containing 140 mmol/L NaCl and 500 mmol/L urea has an *osmolarity* of 780 mosm/L and an *osmolality* of 751 mosm/kg H_2O (i.e. 3.7 % lower). This difference partially compensates for the missing solutes and thus contributes to the almost equality of eU_{osm} and mU_{osm}. Another factor is that electrolytes are assumed to be totally dissociated in the eU_{osm} formula. Although the dissociation is high in solutions within the physiological range, it is less than 100 %, thus also contributing to modestly overestimate eU_{osm}.

UCI is a less accurate reflection of mU_{osm} than eU_{osm} because creatinine is known to undergo some secretion as well as some reabsorption along the tubule. The net result of these opposite effects depends on the rate of urine flow (Bouby N. et al. 1996). Our study shows that individual values are fairly dispersed and the correlations between the two variables are not linear. However, when no other approach is available, UCI remains a possible surrogate of urine concentration, provided it is applied to subjects with normal renal function. As clearly demonstrated in the present study, UCI diverges markedly from mU_{osm} in patients with CKD - limiting its use when renal function is impaired and probably also when abnormal handling of creatinine or excessive 24 h intake of creatine are suspected.

A few alternative methods for quantifying urine concentration have been used. Urine density (UD) (or specific gravity) may be evaluated in 7 coloured grades with commercially available dipsticks (Labtix 8SG and Multistix 8SG AMES/Bayer Diagnostics) or evaluated by refractometry using a hand-held refractometer (Pen Urine S.G., Atago, Tokyo, Japan) (Bottin JH. et al. 2016). In the D.E.S.I.R. study (a cohort of the French population), UD was measured with dipsticks in fresh spot morning urine samples from 1604 subjects, and eU_{osm} was calculated (same formula as here) (Roussel R. et al. 2014). Median (IQR) eU_{osm} was 664 (272) mosm/L. UD was well correlated with eU_{osm} ($r = 0.446$, $P < 0.00001$). Another study showed that UD was well correlated with measured U_{osm} but the wide dispersion made it "impossible to use UD as a dependable clinical estimate of U_{osm}" (Souza AC. et al. 2015). Moreover, UD or specific gravity cannot be used if urine contains proteins or glucose (Leech S. et al. 1987).

It is important to note that U_{osm} varies greatly among different subjects, as shown in the four populations of the present study and in a few previous reports (Perucca J. et al. 2007; Perrier

E. et al. 2013). In usual conditions, some subjects produce hypo-osmotic urine while others show U_{osm} up to 1200 mosm/kg H_2O. This wide range of spontaneous U_{osm} is possibly due to large inter-individual variations in the daily solute load (Berl T. 2008), in fluid intake (Perrier E. 2013) and in thresholds for vasopressin secretion and/or thirst that are, in part, genetically determined (Zerbe RL. et al. 1991). Both vasopressin concentration and urine osmolarity are known to differ between sexes. Men have higher vasopressin/copeptin levels (Share L. et al. 1988; Ponte B. et al. 2015; Roussel R. et al. 2016) and higher U_{osm} than women (Perucca J. et al. 2007). This difference is mostly due to the fact that men excrete a larger osmolar load than women with a higher urine osmolality but an approximatively similar 24 h urine volume (Perucca J. et al. 2007; Perinpam M. et al. 2006). Therefore, in studies using these variables, data for the two sexes are often presented separately. We verified here the validity of the two surrogates in each gender. For both genders, the relation between eU_{osm} and mU_{osm} is highly significant and the regression line between these two variables is very close to the identity line. The UCI also reflected this gender difference but tended to underestimate it slightly, possibly because of the known difference in creatinine handling in men and women.

Differences in the usual urine concentration may be associated with the ethnic background. A few studies showed that African Americans tend to concentrate urine about 20 % more than Caucasians and have higher vasopressin levels (Cowley AW. Jr. et al. 1987; Bankir L. et al. 2007; Chun TY. et al. 2008). To our knowledge, very few studies have evaluated other possible differences in usual urine concentration related to habitat or ethnic background (Katz AI. et al. 1965; Kristal-Boneh E. et al.1968; Berlyne GM. et al. 1976; Taylor GO. et al. 1978; Johnson RH. et al. 1988).

The results of the SKIPOGH study illustrate the fact that urine is usually on the average more concentrated during the night than during the day by about 50 - 100 mosm/kg H_2O. Few studies have investigated day and night urine separately (Bankir L. et al. 2008; Kimura G. et al. 2010; Guerrot D. et al. 2011). They showed that the circadian pattern of urine flow rate/urine concentration and/or sodium excretion rate may be disturbed in some subjects. An excessive urine concentration during daytime, limiting sodium and/or water excretion rate, is subsequently compensated at night by the pressure-natriuresis mechanism (Burnier M. et al. 2007; Bankir L. et

al. 2008; Kimura G. et al. 2010; Guerrot D. et al. 2011; Fezeu L. et al. 2014). Accordingly, measurement of urine osmolality in overnight urine samples may not be representative of 24 h urine.

There are several advantages of using surrogates of urine osmolality. Osmometers, based on either freezing point depression or vapor pressure methods, are expensive and rarely equipped with automatic sample changers. Each measurement lasts a minute or two (due to the time needed to freeze or heat the sample, respectively), thus allowing some evaporation if samples are loaded into the changer in advance. We tested the automatic changer and observed that mU_{osm} values in the same sample increased after 10 loads. In studies, involving a large number of subjects in which individual measurements are practically impossible, values may increase artifactually depending on the timing of the measurements. Moreover, osmolality measurements cannot be coupled with measurements of various solutes performed by automatic analyzers; they thus require separate aliquots and time-consuming manipulations. The excellent correlation between eU_{osm} and mU_{osm}, over the whole range of mU_{osm} values, even in CKD, validate eU_{osm} as an appropriate surrogate of mU_{osm}, especially in large cohorts.

Urine electrolytes are often available in epidemiological studies, but urea, needed for the calculation of eU_{osm}, is less frequently measured. When new measurements are initiated on previously stored samples in order to evaluate the kidney's concentrating activity, authors should consider the respective advantages of measuring either osmolality or urea concentration. Urea is much easier, quicker and cheaper to measure than osmolality. Moreover, it will also provide data for a significant solute in the urinary concentrating process, and allow an indirect evaluation of protein intake.

This study has some limitations. It concerns exclusively subjects of European descent. Studies in subjects of other ethnic backgrounds are required. The possible influence of socio-demographic factors has not been considered. However, we think it is reasonable to assume that the highly significant correlations between eU_{osm} and mU_{osm}, and the relatively good relationships of UCI with mU_{osm} are not dependent upon the population under study and may be extended to all populations, as long as the measurements of sodium, potassium, urea and

creatinine concentrations are performed in appropriately equipped laboratory with rigorous methods.

In summary, the present study validates, in large cohorts, the use of an estimated osmolarity based on the measurement of sodium, potassium and urea, as an excellent surrogate of the measured urine osmolality. It also shows that the urine concentrating index, based on the ratio of creatinine concentrations in plasma and urine, may be used as a relative index of urine concentration only in subjects with normal renal function because of the disturbed handling of creatinine in CKD. In contrast, eU_{osm} is valid whatever the level of renal function. In future epidemiologic studies addressing the influence of vasopressin and urinary concentrating activity, the use of the estimated urine osmolarity should be recommended when the actual urine osmolality cannot be measured.

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VII. Discussion and Outlook

In this work, we validated an in-house immunoassay to establish a standardized procedure for the measurement of uromodulin in the urine. We explored biochemical modifications of this glycoprotein caused by a common variant in the *UMOD* promoter. We used the in-house uromodulin assay in different cohorts in order to identify clinical and genetic factors that modulate uromodulin excretion in the general population. These results contributed to the validation of uromodulin as a biomarker for tubular mass and function in the general population. They also evidenced factors important for its excretion in the urine. Taken together, these results yielded insight into the physiological roles and value of uromodulin as a biomarker.

Despite its long history, a clear understanding of the complex physiological role of uromodulin is still lacking (Devuyst O. et al. 2017). The growing interest in the physiology of uromodulin and its potential value as biomarker required the availability of a robust and faithful method to measure its level in the urine from large cohorts. Thus, a high-throughput and cost-effective immunoassay assay to measure uromodulin were needed, as well as the clarification of issues related to storage and handling of urine samples to perform the assay.

In the first part of this thesis, we described an immunoassay with a linear standard curve over a broad range of values, allowing the detection of uromodulin with high sensitivity and very low inter- and intra-assay variability. After characterizing and validating this assay, we used it as a tool to investigate the stability of uromodulin in human urine under different treatment and storage conditions. We showed that urinary uromodulin levels were significantly decreased by centrifugation (~30 %) which might be clinically misleading in some patients (Rampoldi L. et al. 2011; Bollée G. et al. 2011; Dahan K. et al. 2003; Bleyer AJ. et al. 2004). On the other hand, we showed that vortexing is necessary since it helps to recover uromodulin that tends to aggregate (Zhou H. et al. 2011) and to get trapped in the cell debris (Uto I. et al. 1991). Conflicting results were reported concerning sample treatment prior to dosage in order to solubilize uromodulin (Akesson I. et al. 1978; Uto I. et al. 1991; Torffvit O. et al. 1992; Kobayashi K. et al. 2001; Dawney AB. et al. 1982). Knowing that some of the employed treatments could affect the binding of uromodulin to the ELISA capture antibody we demonstrated that diluting urine sample with deionized water yields similar results as dilution with TEA buffer.

Studying the stability of uromodulin under different storage condition is crucial, particularly when analyzing a large number of samples from multicentric cohorts. Prior studies regarding the influence of storage duration and temperature on uromodulin levels were conflicted by small sample size (Akesson I. et al. 1978; Uto I. et al. 1991; Torffvit O. et al. 1992; Kobayashi K. et al. 2001) and lack of normalization for urinary creatinine (Waikar SS. et al. 2010; Ortiz A. et al. 2011). We showed on a large number of samples that indexed uromodulin levels were influenced by short (1 week) and long storage period independent of temperature (room temperature, 4 °C or -20 °C) revealing a significant decrease in uromodulin levels. These results were in line with the observations of Kobayashi (Kobayashi K. et al. 2001). The degradation effect can be partially mitigated by adding protease inhibitors to samples stored at -20 °C. We observed a negligible and non-significant decrease in the unindexed and indexed uromodulin values in samples that were stored at -80 °C for 4 months. In contrary, a significant decrease in unindexed uromodulin levels was observed after 8-month storage at -80 °C. Moreover, freezing and thawing samples on ice up to 5 times did not affect the stability of uromodulin stored at -80 °C.

These results established a robust immunoassay, which displays a wide detection range of uromodulin levels, together with low variability and low cost. They proved that reliable measurement of uromodulin could be obtained when urine samples are stored at -80 °C and analyzed within 3 months with vortexing and using only water for dilution. This procedure is suitable for high-throughput investigations of uromodulin and its validation as a biomarker for renal function and risk of CKD.

In the second part of the thesis, we investigated the effect of a common variant (rs1297707) in the promoter region of *UMOD* on the levels of uromodulin in urine and blood as well as on the biochemistry of this protein. The regulatory effect of the *UMOD* promoter variants on the transcription and urinary levels of uromodulin is well established (Trudu M. et al. 2013; Olden M. et al. 2014). However, a possible influence of these variants on the biochemical features of uromodulin and on its circulating levels is still unknown.

Several studies corroborated that the major alleles (risk alleles) of GWAS-identified common variants in the promoter region of *UMOD* are in total LD with the variant rs12917707 and are associated with increased risk for several renal disorders in the general population (chronic kidney disease, kidney stones and hypertension) (Köttgen A. et al. 2009; Köttgen A. et

al. 2012; Padmanabhan S. et al. 2010; Reznichenko A. et al. 2012). Moreover, these risk alleles were correlated with elevated urinary uromodulin levels (Olden M. et al. 2014) indicating that elevated uromodulin levels were linked to higher predisposition to certain renal disorders. In this work, carried out in Swiss cohorts, we showed that the risk allele G of variant rs12917707 resulted in higher uromodulin levels in comparison to the minor allele, independent of normalization to creatinine. We also showed that the *UMOD* variant rs12917707 regulated changes in plasma uromodulin levels: Higher circulating uromodulin levels were observed in subjects harbouring the major allele (G) when compared to age and gender-matched subjects harbouring the minor allele (T). Of note, plasma uromodulin levels were approximately 1000-fold lower than the urine levels. We were the first to investigate modulators of circulating uromodulin levels by showing plasma uromodulin together with urine uromodulin in the same population with regards to the genotype of *UMOD* variant. In a recent study, Graciela et al. (Graciela E. et al. 2017) explored plasma uromodulin as a biomarker for cardiovascular diseases and reported higher plasma uromodulin levels in the presence of risk allele for rs12017707. However, they did not analyze urine uromodulin levels in the same population. We suggested that high levels of circulating and urine uromodulin levels associated with the *UMOD* risk allele could be an early indicator of tubular dysfunction, which may precede damage in the tubular interstitium.

We next tested whether *UMOD* promoter variants, which are located in a strong LD block comprising exons 3, 4 and 6, are also changing the glycosylation pattern of uromodulin. Uromodulin harbours seven sites for N-glycosylation, a post-translational modification that is essential for its functions, such as binding to type I fimbriated *Escherichia coli* (Bates JM. et al. 2004). Possible genotype-related changes in glycosylation patterns might affect the interactions of uromodulin with uropathogenic *E. coli*, thus modulating the propensity for urinary tract infections (UTIs). Based on age- and gender-matched urine samples, we identified differences in immunoreactivity as well as in isoelectric point (IP) among genotypes: The minor allele T samples had lower immunoreactivity and higher IP when compared to sample from the major allele G. However, we were able to eliminate these discrepancies with de-N-glycosylation which hinted towards divergences in N-glycan composition. We confirmed the effect of *UMOD* genotype through completing N-glycan profiling by MS-MALDI in matched urine samples. The differences in N-glycosylation were not caused by modifications in the coding sequence of

UMOD (Köttgen A. personal communication). Instead, we hypothesized that alteration in the glycan composition might reflect a mechanism of adaptation by the cell machinery to the increased production of uromodulin when the risk allele is present. Considering the limited number of matched samples analyzed, further studies are necessary to elucidate the effect of *UMOD* variant on uromodulin N-glycosylation and its potential consequences on the biology of the protein in the urine.

In the third part of the thesis, we investigated the clinical and genetic factors modulating uromodulin excretion in the general population. These investigations were carried out in three large cross-sectional studies from Switzerland (SKIPOGH, CoLaus) and Canada (CARTAGENE). We found a positive correlation between uromodulin excretion levels and renal mass, renal volume, eGFR and urine parameters, indicating that uromodulin levels reflect tubular mass and tubular function. Urine uromodulin was also associated with urine osmolality, parameter that reveal tubular activity, which supports uromodulin as a good marker of tubular activity. These outcomes supported findings from previous studies (*in vitro* and *in vivo*) that showed uromodulin as a modulator of transport processes in the TAL by enhancing ROMK activity and increasing the phosphorylation of NKCC2 processes thus affecting urine concentrating ability (Renigunta A. et al. 2011; Mutig K. et al. 2011). Conversely, age and glycosuria were negatively associated with uromodulin levels, which might also reflect tubular damage. It has been shown that glycosuria modulates the TAL activity consequently uromodulin excretion (Riazi S. et al. 2006). Our results were in line with a study on CKD patients with diabetes but without glomerular dysfunction (Möllsten A. et al. 2010). Validation of these findings in a large diabetic-specific study is necessary. Furthermore, genetic variants positioned in the *UMOD* promoter and the *PDILT* genes were implicated in regulating the urinary levels of uromodulin.

The positive association we identified among uromodulin levels and kidney structural parameters, functional indicators as well as urinary electrolytes and osmolality on one hand and the negative association with age and glycosuria on the other hand, validated uromodulin as a biomarker for tubular mass and tubular function in the general population. These solid associations specifically with urine electrolytes, eGFR and osmolality were consistent whether in 24 h or spot urine. Our findings complemented previous studies that were conducted in specific groups of patients with renal diseases such as autosomal dominant polycystic kidney disease,

diabetic nephropathy, and tubulointerstitial nephritis. This study has many advantages: studied populations had a large sample size in addition to the detailed information about participants. Uromodulin measurements, as well as urine parameters, were carried out in one center using a well-established method. Renal length and renal mass measurements were achieved using standardized protocol. As limitations, this study lacked the ethnic diversity and the limitation in assessing causal conclusions because these are cross-sectional studies.

In a final part, we tested the validity of two surrogates for urine osmolarity: the estimated urine osmolarity (eUosm) which is determined from the concentration of three main urinary parameters: sodium, potassium and urea (Perucca J. et al. 2007; Zittema D. et al. 2014), and the urine concentrating index (UCI), which depends on the handling of creatinine by the kidney (Perucca J. et al. 2007; Bankir L. et al. 2007). Since these parameters have not been validated, we assessed eUosm and UCI compared to measured Uosm (mUosm) in large population-based and CKD cohorts. We also studied the effect of gender, age and sample type (spot or 24 h, day and night) on these surrogates.

We showed that eUosm is a good surrogate for mUosm with a difference ± 5 %. These findings were independent of gender, urine type (day and night) and renal function (measured with eGFR). The differences between eUosm and mUosm were caused by solutes that were not considered when using the eUosm formula and also because in this formula electrolytes were considered to be totally dissociated whereas in reality, this dissociation is less than 100 % – both factors leading to a slight overestimation of eUosm. These results were in line with results from the D.E.S.I.R. study in which urine density (UD) correlated significantly with eUosm in fresh spot morning urine samples from 1,604 subjects ($r = 0.446$, $P < 0.00001$) (Roussel R. et al. 2014). Another study showed that UD correlated with measured Uosm but with a wide dispersion (Souza AC. et al. 2014). Our results also showed that UCI is an acceptable surrogate for urine concentration showing non-linear correlation with mUsom. UCI was less accurate than eUosm in reflecting mUosm since urine creatinine values depend on the urine flow rate that affects creatinine secretion and reabsorption along the tubule (Bouby N. et al. 1996). Nevertheless, UCI can still be considered as surrogate for urine osmolarity when other approaches are not available. This measurement deviated noticeably from mUosm in patients with CKD, which limits its use to subjects with normal renal function.

We observed a large variability in Uosm levels among subjects from the four studied populations. This variability is induced by the inter-individual variations in daily solute and fluid intake (Pierre E. et al. 2013; Berl T. et al. 2008) also by vasopressin secretion and/or thirst that are genetically determined to some degree (Zerbe RL. et al. 1999). Besides vasopressin concentration and urine osmolarity differ between men and women: men have higher vasopressin/copeptin levels (Share L. et al. 1988; Ponte B. et al. 2015; Roussel R. et al. 2016) and higher Uosm compared women (Perucca J. el al. 2007). The significant correlation between eUosm and mUosm that covered a wide range of mUosm values, even in CKD, corroborated eUosm as a suitable surrogate for mUosm. Particularly surrogates are useful in investigations of large cohorts, where separate measurements tend to overestimate Uosm because of time thus making these measurements practically impossible in addition to time-consuming manipulations.

This study had a limitation: it concerned exclusively subjects of European origin. Studies in subjects with different ethnic backgrounds are necessary.

Conclusion: In this thesis, we provided a robust and cost-effective assay for determination of uromodulin levels in the urine, with appropriate validation of sampling and experimental procedures. Based on this assay, we could substantiate the value of uromodulin as a biomarker for kidney tubular mass and function in the general population. We also provided novel indication that *UMOD* promoter variants not only influence the absolute levels of uromodulin production and secretion, but also its glycosylation pattern. These findings need further explorations to identify the mechanisms regulating the production and biochemical maturation of uromodulin, and its release at the apical and basolateral side of the cells lining the TAL in the kidney. These studies are important to better understand the relationship between uromodulin structure and the transport activity in the TAL and, more generally, the physiological role of this protein.

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VIII. Curriculum vitae

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Education

2011 - 2017
PhD student: "Uromodulin: Biomarker for tubular function**"**In the group of Professor Dr. Olivier Devuyst – Mechanisms of Inherited Kidney Disease at University of Zurich, Institute of Physiology – Zurich – Switzerland.

2007 - 2011
Masters in Molecular Biology: "Parental Consanguinity and Family History of Coronary Artery Disease Strongly Predict Early Stenosis" At the Lebanese American University – Byblos – Lebanon.

1998 - 2002
Bachelor of Science in Medical Laboratory: "Genetic susceptibility to Diabetes Type I: HLA alleles." At the Lebanese University – Faculty of public health at Beirut – Lebanon.

1989 - 1989
General Secondary Diploma: Scientific section equivalent to "Matura" at "Notre Dame des Carmelites" – Beirut – Lebanon.

Work Experience

2007 - 2011:
Research Associate/Project coordinator: Molecular basis of common diseases (Coronary Artery Disease) Lebanese American University School of Medicine – Byblos – Lebanon

2003 - 2007:
Research Assistant: Clinical trials and medical research at American University of Beirut Medical Center (AUBMC).

Teaching experience

2012 - 2014:
Teaching assistant at University of Zurich: First and second year medical students in the following: Central Nervous System, ECG, signal transduction and respiration.

2008 - 2010:
Practicum lecturer at Lebanese American University: General Biology course for biology students

Awards

2012: Travel grant from International Society of Nephrology 2012

Poster presentations

2016: 6th Kidney – Control of Homeostasis Retreat
2015: National Centres of Competence in Research Kidney.CH Retreat
2014: National Centres of Competence in Research Kidney.CH Retreat
2013: National Centres of Competence in Research Kidney.CH. Retreat
2013: National Centres of Competence in Research Kidney.CH. Site visit in Zurich
2012: National Centres of Competence in Research Kidney.CH. Retreat
2012: SSN/SGN Annual Meeting Zurich
2012: International Society of Nephrology. Ann Arbor, Michigan, USA

Publications

Youhanna S., Bankir L., Jungers P., Porteous D., Polasek O., Bochud M., Hayward C., Devuyst O. Validation of Surrogates of Urine Osmolality in Population Studies. *Am J Nephrol*. 2017; 46 (1): 26-36.

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